REMARKS/ARGUMENTS

In view of the foregoing amendments and following remarks, favorable reconsideration of the pending claims is respectfully requested.

Applicants thank the Examiner for indicating that Claims 40-47, 49-53, and 67-78 are allowable.

Status of the Claims

Claims 35-53, 55-57, and 67-79 are pending.

By way of this amendment, Claims 39 and 79 have been cancelled.

Claim 35 has been amended to recite that the complex Tat/ligand or artificial variant of an HIV Tat protein or of a Tat fragment is capable of inducing an anti-Tat specific humoral and/or cellular immune response in humans. Claims 44 and 52 have similarly been amended. The amendments are supported, for example, by the definitions on page 7, line 38 to page 8, line 2. No new matter has been added.

Rejections Under U.S.C. § 112 second paragraph

Claim 39 has been rejected as being indefinite under U.S.C. § 112 second paragraph. As noted above, Claim 39 has been cancelled and therefore this rejection is now moot. Withdrawal of the rejection is therefore respectfully requested.

Prior Art Rejections

Claims 35-37, 42, and 48 have been rejected under 35 U.S.C. § 102(b) as being anticipated by Hakansson and Caffrey. Claims 55-57 have been rejected under 35 U.S.C. § 103(a) as being unpatentable over Hakansson and Caffrey in combination with Lindblad. Applicants respectfully traverse these rejections.

The Applicant would like to point out to the Examiner that in the present Office Action, there is a discrepancy between the claims which are rejected and the claims which are considered as allowable subject matter. Claims 35-37, 42 and 48 are rejected in section 7 (beginning of section 7, page 4 and end of section 7, page 5) and claims 40-47 and 67-68 (which include claim 42 but not claim 38) are considered as allowable subject matter in section 9 (page 6) of the Office Action. However, the rejection set forth in section 7 is based on arguments which clearly apply only to claim 38 and not to claim 42

(seventh line of section 7). Therefore, the applicant considers that there is an error in the claims rejected in section 7 and that the rejection in section 7 is not directed to claims 35-37, 42 and 48, but to claims 35-38 and 48 instead.

Briefly, an objective of the claimed invention is to provide a composition comprising a Tat antigen which is more immunogenic than the compositions comprising a Tat antigen of the prior art. In particular, the claimed invention provides a stabilized Tat antigen which is more immunogenic than the Tat antigens of the prior art. The stabilized Tat antigen according to the invention comprises a complex Tat/non-metal ligand of Tat, a modified Tat wherein one or more cysteines are modified with a hydrophobic group and/or substituted with a hydrophobic amino acid, or a complex between said modified Tat and a non-metal ligand of Tat.

Claims 35-38 and 48 were rejected as being anticipated by Hakansson and Caffrey. This rejection is obviated by amendment and traversed in part. The new claims are limited to an HIV immunogenic composition comprising a complex Tat antigen/ligand wherein the Tat antigen comprises an HIV Tat protein or a Tat fragment of at least 11 amino acids capable of inducing a specific humoral and/or cellular response in a human, *i.e.* a Tat fragment of at least 11 amino acids which is immunogenic in humans. In view of the amendments to the claims and the remarks provided below, it is respectfully submitted that the Hakansson and Caffrey does not disclose each and every element recited in the claims and therefore the claimed invention is not anticipated by the cited art.

A Tat peptide which is immunogenic peptide in humans comprises at least a Tat CD4+ T-cell epitope able to induce a Tat specific CD4+ T-cell response in humans since CD4+ T cells are required to induce a specific humoral and cellular immune response to an antigen.

An antigen is immunogenic when it is able to induce a B-lymphocyte response, or a CD4+ auxiliary T-lymphocyte response or a CD8+ cytotoxic T-lymphocyte response. It has been known for a long time that B-lymphocyte stimulation requires the help from CD4+ auxiliary T-lymphocytes (Mitchison NA, 1971, The carrier effect in the secondary response to hapten-protein conjugates, Eur. J. Immunol., 1 :10-17 and 1 :18-27;

Lanzavecchia A, 1985, Antigen-specific interaction between T and B cells, Nature, 314 :537-539; Francis MJ et al., 1987, Non-responsiveness to a foot and mouth disease virus peptide overcome by addition of foreign helper T-cell déterminants. Nature, 330:168; Abstracts herewith attached in ***Annex I***). In addition, it is well-known that the help from CD4+ auxiliary T-lymphocytes is also required for an efficient stimulation of CD8+ cytotoxic T-lymphocytes (Bevan MJ, 2004 Helping the CD8+ T-cell response, Nat. Rev. Immunol. 4:595–602 (Abstract herewith attached in ***Annex I***); Novy P, Quigley M, Huang X et al., 2007, CD4 T cells are required for CD8 T cell survival during both primary and memory recall responses. J Immunol 179:8243–8251; *Annex II*).

This forms the basis for an absolute need to include a region from the pathogen comprising at least a T epitope recognized by specific CD4+ auxiliary T-lymphocytes, to obtain a vaccine which is an efficient immunogen.

Hakansson and Caffrey disclose a **PG-TTD fusion protein** comprising : (a) the IgG binding domain of streptococcal protein G (PG), (b) a six-residue linker region, (c) the Tat Transduction Domain (TTD; 11-residue basic region (amino acids 47 to 57) of HIV-1 Tat), and (d) a four residue cloning residue at the C-terminus (see the first paragraph of the Material an Methods section spanning pages 8999 and 9000 which is cited by the Examiner in section 7, page 4 of the Office Action).

Hakansson and Caffrey teach that the PG-TTD fusion protein binds to heparin (see all the document which discloses the PG-TTD fusion protein in the free state or in complex with heparin (heparin-bound state)).

Therefore, Hakansson and Caffrey disclose a composition comprising a **PG-TTD fusion protein** complexed with a 6000 Da MW heparin molecule.

The TTD peptide (Tat 47-57) is not immunogenic in humans since it is not able to induce a CD4+ T-cell response in HIV-seronegative or HIV-infected humans, exhibiting a diversity of HLA II molecules (Castelli *et al.*, Eur. J. Immunol., 2008, 38, 2821-2831: *Annex III*; Blazevic *et al.*, J. Acquir. Immune Defic. Syndr., 1993, 6, 881-890; *Annex IV*).

In nine HIV-seronegative donors, who exhibited a diversity of HLA II molecules, including all HLA-DR molecules, the CD4+ T-cell response is directed mainly towards Tat 41-55 (immunoprevalent CD4+ T-cell epitope), and to a lesser extent towards Tat 24-

38 and Tat 30-44 (Table 3 and page 2822, 2nd column, 2nd paragraph of Castelli *et al.*). The other peptides tested: Tat 1-15, 6-20, 9-23, 34-48, 45-59 which includes Tat 47-57 (TTD peptide), and 65-79 do not induce a CD4+ T-cell response.

In another study (Blazevic *et al.*), three Tat peptides, 17-32, 33-48 and 65-80 were recognized by the CD4+ T-cells from fourteen HIV-positive patients.

Therefore, Hakansson and Caffrey do not disclose a composition comprising an immunogenic Tat peptide complexed with heparin as required in the new claim 35.

Furthermore, Hakansson and Caffrey do not teach any HIV antigen, immunogen or immunogenic composition.

The Examiner argues (section 7, page 4) that "*it is well-known in the immunological arts that heparin per se is immunogenic*".

However, the present invention does not relate to an immunogenic composition but to an HIV immunogenic composition (see claim 1 and the overall specification).

An HIV immunogenic composition means a composition which induces an immune response which is specifically directed to HIV.

In the case of the present invention, the HIV-specific immune response is directed against the HIV Tat antigen (see the overall specification of the present application and in particular page 7, line 1 to 4 and the definitions page 7, line 36 to page 8, line 2).

Therefore, the fact that heparin *per se* is immunogenic is not relevant for the claimed composition which comprises a Tat antigen which is more immunogenic than the Tat antigens of the prior art, as mentioned above.

Hakansson and Caffrey relate to intracellular drug delivery using TTD-fusions (see the end of the first paragraph of the introduction page 8999).

Therefore, Hakansson and Caffrey relate to a technical field which is distinct and remote from the field of anti-HIV vaccine (induction of a protective immune response against HIV infection and AIDS).

The aim of the work reported by Hakansson and Caffrey is to understand how proteins containing TTD translocate across biological membranes (TTD-mediated membrane translocation) and to determine the implication of the cell-surface heparan sulfate in the TTD-mediated internalization (see in particular the introduction page 8999).

To this end, a PG-TTD was constructed and the structural and dynamic properties of the PG-TTD in the free and heparin bound states were studied since heparin is a naturally occurring analogue of heparan sulfate which can be used as a model of heparan sulfate found at the cell surface of many cell types (see the end of the introduction page 8999, the beginning of second paragraph page 9002 and the beginning of the second paragraph of the discussion page 9004).

Hakansson and Caffrey neither disclose nor suggest an HIV Tat immunogen, Hakansson and Caffrey teach only that Tat Transduction Domain (TTD) is able to mediate membrane translocation of cargo proteins which are fused to TTD (TTD-fusions).

Hakansson and Caffrey teach that the TTD moiety in the context of a cargo protein is in an extended conformation and relatively dynamic **in the free state**. In the same paragraph, Hakansson and Caffrey teach that the lack of regular secondary structure of TTD may be an inherent property for membrane translocation (1st paragraph of the discussion page 9004), *i.e.* a property of Tat which is different and totally unrelated to Tat immunogenicity.

The Examiner argues also that (section 7, page 4) "*according to Hakansson and Caffrey, the 11 amino acid Tat moiety of the so called PG-TTD fusion protein is expected to be in an extended form and at least partially exposed so as to remain immunogenic*".

However, the Examiner is just making a simple assertion. The Examiner does not mention any specific passage from Hakansson and Caffrey supporting this assertion because there is none.

Caffrey mentions only: "*Thus it can be inferred that heparin does not induce a change in secondary structure of PTD and that TTD remains in an extended conformation*" (page 9003, 2nd colum, end of first paragraph) and "*In the present study structure of the TTD moiety has been modelled in an extended conformation, based on the secondary NMR chemical shifts, NOEs and CD of TTD in the presence of heparin*" (page 9005, 1st column, end of 2nd paragraph).

Firstly, the fact that TTD remains in an extended conformation does not imply necessarily that TTD is accessible. This is indeed confirmed from the next sentence from

page 9005, 1st column, end of 2nd paragraph which mentions that "*On the basis of the ITC results*, ***an electrostatic interaction between positively charged TTD and negatively charged heparin is highlighted by their respective electrostatic potentials***". TTD sequence: YG**RKKRR**Q**RRR** (shown on page 8999, 1st column, beginning of 1st paragraph) comprises 8 positively charged residues (bold underlined). These data demonstrate that the majority of TTD amino acid side chains (8 out of 11) interact with heparin and are thus not accessible. Out of the three remaining amino acid residues, two only (Y and Q) have accessible side chains. These two residues are not sufficient for specific recognition by antibodies.

Secondly, the accessibility of an antigen to antibody recognition relates to its antigenicity, which is mediated by B-lymphocytes.

The fact that a protein is antigenic is not relevant for its immunogenicity, which requires in the case of the Tat vaccine of the present invention, a Tat CD4+ T-cell epitope able to induce a Tat specific CD4+ T-cell response in humans (see the above exampled basis of protein immunogenicity).

As discussed above, the TTD is not immunogenic in humans since it is not able to induce a CD4+ T-cell response in HIV-seronegative or HIV-infected humans.

For these reasons, the Examiner is simply making an incorrect assertion which cannot be supported and is in fact contrary to the teaching of Hakansson and Caffrey.

Hakansson and Caffrey teach to prepare **TTD fusions** to deliver a wide variety of diagnostic and therapeutic agents to diverse cell types (end of the first paragraph of the introduction page 8999).

Hakansson and Caffrey teach that TTD binding to heparan sulfate present on cell surfaces is essential for TTD-mediated membrane translocation (see figure 6 and the 2nd paragraph of the first column, page 9005).

In addition, Hakansson and Caffrey teach that heparin is an heparan sulfate analogue which binds to TTD with high affinity (see the beginning of the second paragraph page 9002 and the beginning of the second paragraph of the discussion page 9004).

Therefore, the skilled man with the teaching of Hakansson and Caffrey would neither have modified the PG-TTD to prepare a PG-TTD/heparin complex since the high affinity binding of heparin to TTD would inhibit TTD binding to cell surface protean sulfate and thus prevent TTD-mediated cell entry.

Furthermore, it is well-known in the immunological arts that the immunogenicity of a therapeutic protein neutralizes its therapeutic effect (see for example De Groot and Scott, TRENDS in Immunology, 2007, 28; *Annex V*).

For this reason, one with ordinary skills in the immunological arts, would neither have prepared fusion proteins wherein the TTD is replaced with larger fragments of the HIV Tat protein which are immunogenic such as the full length Tat protein or the fragment Tat 1-57 which includes the immunoprevalent CD4+ T-cell epitope Tat 41-55, as well as CD8+ T-cell and B-cell epitopes (see claims 35 and 44).

In addition, the skilled man with the teaching of Hakansson and Caffrey would neither have prepared a composition comprising a Tat fragment with free N-terminus and C-terminus (not fused to a protein) since Hakansson and Caffrey teach to prepare **TTD fusions** to deliver a wide variety of diagnostic and therapeutic agents to diverse cell types.

By contrast, in the present invention, the inventors have shown that, surprisingly, Tat/heparin complexes induce anti-Tat antibodies which are at least ten times higher in animals (factor of 10 to 35) than those obtained with Tat in the free state (examples 3, 4, 7 and figures 4, 9, 12, 13, 16 and 17). In addition, the inventor have shown that the interaction of Tat with heparin does not mask certain antigenic sites of Tat and unmask others since the antigenic specificity of the anti-Tat antibodies produced by immunization with Tat/heparin complexes is similar to that produced by immunization with free Tat (example 3 page 34, line 18 to page 35 line 7 and figure 5).

Accordingly, this rejection cannot be sustained because the cited art does not disclose each and every element of the claimed invention, or suggest or provide an enabling disclosure, or provide a reasonable expectation of success for the present invention. Withdrawal of the rejection is therefore respectfully requested.

Claims 55-57 were rejected under 35 USC § 103(a) as being unpatentable over Hakansson and Caffrey as applied to claims 35-38 and 48 and in further in view of Lindblad (2004). This rejection is not sustainable over Hakansson and Caffrey for the reasons discussed above. Lindblad was cited as disclosing aluminium adjuvants. However, Lindblad does not suggest the elements missing from the primary reference, such as an HIV immunogenic composition comprising a complex Tat antigen/ligand wherein the Tat antigen comprises an HIV Tat protein or a Tat fragment of at least 11 amino acids capable of inducing a specific humoral and/or cellular response in humans, *i.e.* a Tat fragment of at least 11 amino acids which is immunogenic in humans. Accordingly, the combination of Hakansson and Caffrey and Lindblad also fails to disclose or suggest each and every element recited in Claims 55-57, and therefore the Examiner has failed to establish a *prima facie* case of obviousness.

Accordingly, this rejection cannot be sustained. Withdrawal of the rejection is therefore respectfully requested.

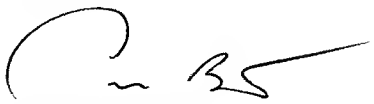
In view of the foregoing amendments and remarks, it is respectfully submitted that the rejections under 35 USC § 102 and 103 have been overcome and that the pending claims are in condition for allowance.

Conclusion

In view of the claim amendments and the foregoing comments and accompanying evidence, it is submitted that all outstanding issues have been overcome and the claims of this application are in condition for immediate allowance. Favorable reconsideration by the Examiner and formal notification of the allowability of the claims are solicited.

It is not believed that extensions of time or fees for net addition of claims are required, beyond those that may otherwise be provided for in documents accompanying this paper. However, in the event that additional extensions of time are necessary to allow consideration of this paper, such extensions are hereby petitioned under 37 CFR § 1.136(a), and any fee required therefor (including fees for net addition of claims) is hereby authorized to be charged to Deposit Account No. 16-0605.

Respectfully submitted,



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Annex I

Eur J Immunol. 1971 Jan;1(1):10-7.

The carrier effect in the secondary response to hapten-protein conjugates. I. Measurement of the effect with transferred cells and objections to the local environment hypothesis.

Mitchison NA.

Abstract

A carrier effect is obtained typically when a hapten-protein conjugate is injected into an animal which has previously been primed with the same hapten conjugated to another carrier protein. Under these circumstances the anti-hapten secondary response is usually less than that which would have been obtained had the animal been injected with a conjugate prepared with the same carrier as that originally used for priming. Attempts have been made to account for the phenomenon in terms of the local environment hypothesis, which assumes that the receptor on immunologically competent cells recognises the hapten jointly with the area on the complete antigen which surrounds it. Alternatively the phenomenon can be accounted for by the hypothesis of cooperation, which assumes that the antigen is recognised by two receptors, one directed to the hapten and the other to a determinant on the carrier protein.

Methods are described which enable carrier effects to be studied quantitatively in mice. They involve a cell transfer system in which cell suspensions prepared from large numbers of donors are distributed among irradiated syngeneic recipients. In these recipients the transferred cells can be made to perform a secondary response by appropriate antigenic stimulation. The response is monitored by binding tests in which the capacity of serum to bind highly radioactive haptens or proteins is measured. The haptens employed in this system are NIP (4-hydroxy-5-iodo-3-nitro-phenacetyl-) and DNP (2,4-dinitrophenyl-) and the proteins comprise chicken γ-globulin, bovine serum albumin, human serum albumin, ovalbumin, bovine γ-globulin, keyhole limpet hemocyanin and mouse γ-globulin.

A carrier effect was regularly obtained when the proteins were tested against one another as carriers, for priming and for the secondary response. The effect could best be measured by comparing the relative potencies in the secondary response of the homologous conjugate (*i.* e. one with the carrier which had originally been used for priming) with heterologous conjugates (*i.* e. ones with new carriers). In this way the intrinsic potency of the individual protein could also be measured and allowance made for it in calculating the magnitude of the carrier effect. An average carrier effect of one thousand-fold relative potency was obtained. Priming by NIP-ovalbumin or NIP-chicken γ-globulin with secondary stimulation by NIP-bovine serum albumin (or the corresponding DNP conjugates) could be identified as the combination best suited to further study.

Support for the cooperation hypothesis, particularly for that version of the hypothesis which postulates that recognition of carrier determinants allows an antigen-concentrating mechanism to operate, could be found in the parallel slopes of the dose-response curves obtained with homologous and heterologous conjugates. On the other hand the local environment hypothesis failed to pass either of the tests to which it was subjected. One, the weaker, was to compare haptens with and without spacer groups inserted between themselves and the carrier protein, in the expectation that spacers might reduce the local environment contribution: no difference could in fact be detected. The other, the stronger, was to attempt to inhibit the response with an excess of carrier protein even though the anti-hapten antibody had no detectable affinity for the carrier: such inhibition could regularly be obtained.

Annex I

Eur J Immunol. 1971 Jan;1(1):18-27.

The carrier effect in the secondary response to hapten-protein conjugates. II. Cellular cooperation.

Mitchison NA.

Abstract

The adoptive secondary response of mice to conjugates of NIP (4-hydroxy-5-iodo-3-nitro-phenacetyl-) and DNP (2,4-dinitrophenyl-) is here used to elucidate the mechanism of cellular cooperation. The framework into which the experiments fit can be formulated as follows. Priming immunization raises a crop not only of specific antibody-forming-cell-precursors (AFCP) but also of specific helper cells. Upon secondary stimulation the helper cells serve a role as handlers or concentrators of antigen, thus enabling AFCP which would otherwise be incapable of reacting to initiate antibody synthesis. In this act of cooperation both cells recognise antigen; in the system examined here the helpers recognise carrier determinants and the AFCP recognise either the hapten or other carrier determinants.

The first aim of the experiments was to raise populations of helpers and AFCP of distinguishable specificity. Mice were primed with NIP-Ovalbumin (OA) mixed with chicken γ-globulin (CGG) and bovine serum albumin (BSA); in comparison with controls primed with unmixed NIP-OA, their cells after transfer were relatively more sensitive to secondary stimulation with NIP-CGG or NIP-BSA and similar findings were obtained in cross-checks of these carriers. For reasons which are not entirely clear, non-transferred cells did not show the same effect. In further experiments cells primed with one conjugate (e. g. NIP-OA) were mixed with cells primed with another protein (e. g. BSA), transferred and challenged with the hapten conjugated to the second protein (i. e. NIP-BSA). In comparison with controls lacking the protein-primed cells, the mixture regularly showed greater sensitivity to stimulation. NIP and DNP were tested in many of the possible combinations with BSA, OA and CGG with the same result. The mixture system was used in the further analysis.

Tests with allotype-marked protein-primed cells showed that these cells did not participate in the production of the anti-hapten antibody and could therefore properly be regarded as helpers. Tests of specificity showed that physical union of the hapten and carrier were required: cells primed with BSA, for example, would not help NIP-OA-primed cells to make a response to NIP-HSA even when stimulated at the same time with BSA. Transfer of less than one-tenth of the spleen gives a maximum helper effect, whereas AFCP activity continues to rise as larger numbers of cells are transferred. Helper cells are therefore normally present in excess.

Helper activity is more resistant than AFCP activity to irradiation, drugs and semi-allogeneic cell transfer across an H-2 barrier. This suggests that helper cells play a relatively passive role in the immune response.

Several observations indicate that helper cells are thymus-derived mediators of cellular immunity. Passively transferred antibody did not substitute for helper cells. After immunization helper activity developed faster than AFCP activity. Spleen cells obtained from lethally-irradiated, thymocyte-repopulated, immunized donors provided help. Cells from the thymus-derived fraction of thymus/marrow chimeras also appear to provide help.

Thus, the hapten-carrier cooperative response maps onto the well-established synergy of thymus and marrow in the response to foreign erythrocytes.

Annex I

Nature. 1987 Nov 12-18;330(6144):168-70.

Non-responsiveness to a foot-and-mouth disease virus peptide overcome by addition of foreign helper T-cell determinants.

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Abstract

Study of the immune response to synthetic antigens has shown that uncoupled peptides can realize their potential as vaccines only if they contain domains that react with helper T-cell receptors and Ia antigens in addition to antibody binding sites. Here we consider whether genetically restricted non-responsiveness to an uncoupled peptide could be overcome by synthesizing a peptide with an additional helper T-cell epitope from a different protein. We demonstrate that H-2d mice, which are non-responders to the 141-160 VP1 peptide of foot-and-mouth disease virus (FMDV), can be converted into responders by immunization with peptides containing the FMDV sequence with defined 'foreign' helper T-cell determinants from ovalbumin or sperm whale myoglobin. Furthermore, the virus-neutralizing activity of the antibody raised against peptide was dependent on the determinant used. Thus, FMDV peptides with the added sequences 323-339 from ovalbumin and 132-148 from sperm-whale myoglobin elicited a high degree of neutralizing activity in B10.D2 mice. The sera from mice which received the peptide with the added sequence 105-121 from sperm whale myoglobin did not neutralize the virus, although they had high levels of anti-141-160 FMDV peptide activity. Our data indicate that the T-cell help given by the 'foreign' epitopes is B-cell clone specific. These results are likely to have important implications for the design of peptide vaccines.

Annex I

Nat Rev Immunol. 2004 Aug;4(8):595-602.

Helping the CD8(+) T-cell response.

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Cytotoxic T lymphocytes (CTLs) that express the CD8 co-receptor are the guided missiles of the immune system. They express clonally distributed receptors for foreign antigen, undergo marked proliferation in response to infection and kill any cell that expresses their target antigen. When an infection is cleared or brought under control, the progeny of these cytolytic effectors are retained as an essential component of immunological memory. As I discuss here, similar to other aspects of immunity, their clonal expansion and survival depend on the activity of $CD4^+$ T cells, although the mechanism(s) of 'help' for CTL responses is still debated.

ANNEX II

The Journal of Immunology

CD4 T Cells Are Required for CD8 T Cell Survival during Both Primary and Memory Recall Responses

Patricia Novy, Michael Quigley, Xiaopei Huang and Yiping Yang

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CD4 T Cells Are Required for CD8 T Cell Survival during Both Primary and Memory Recall Responses[1]

Patricia Novy,[†] Michael Quigley,[†] Xiaopei Huang,[*] and Yiping Yang[2*†]

The role of CD4 T cell help in primary and secondary CD8 T cell responses to infectious pathogens remains incompletely defined. The primary CD8 T response to infections was initially thought to he largely independent of CD4 T cells, hut it is not clear why some primary, pathogen-specific CD8 T cell responses are CD4 T cell dependent. Furthermore, although the generation of functional memory CD8 T cells is CD4 T cell help dependent, it remains controversial when the "help" is needed. In this study, we demonstrated that CD4 T cell help was not needed for the activation and effector differentiation of CD8 T cells during the primary response to vaccinia virus infection. However, the activated CD8 T cells showed poor survival without CD4 T cell help, leading to a reduction in clonal expansion and a diminished, hut stahle CD8 memory pool. In addition, we ohserved that CD4 T cell help provided during both the primary and secondary responses was required for the survival of memory CD8 T cells during recall expansion. Our study indicates that CD4 T cells play a crucial role in multiple stages of CD8 T cell response to vaccinia virus infection and may help to design effective vaccine strategies. *The Journal of Immunology,* **2007, 179: 8243–8251.**

Adaptive CD8 T cell immunity represents an essential arm of the immune system to protect against many viral and bacterial infections (1, 2). The course of the CD8 T cell response after an acute infection consists of three well-defined phases: clonal expansion of Ag-specific T cells to produce large numbers of effector cells, subsequent contraction of the majority (90–95%) of these effectors via apoptosis, and eventual development of a stable memory population from the surviving cells (1). It has been shown that the clonal expansion and the contraction phases are programmed and that a brief encounter with Ag is sufficient to drive differentiation of naive CD8 T cell precursors into long-lived memory CD8 T cells (3–6). Although initial recognition of peptide-MHC ("signal 1") by the TCR on naive CD8 T cells along with costimulation ("signal 2") provided by mature dendritic cells is critical for induction of CD8 T cell responses following infection, other factors including CD4 T cells, have been shown to play an important role in influencing this process.

The importance of CD4 T cell help in primary CD8 T cell responses in vivo was first demonstrated in immunizations with noninflammatory Ags such as male minor HY Ag and alloantigen Qa-1 (7, 8). Subsequent studies have shown that CD4 T cell help is required for the induction of optimal primary CD8 responses with soluble proteins, tumor Ags, and peptide-pulsed APCs (9–11). It is believed that to prime a CD8 T cell response in the absence of inflammation, APCs such as dendritic cells (DCs)[3] have to be activated by CD4 T cells through CD40-CD40L interactions between DCs and CD4 T cells (12–14). CD4 T cell help may also be provided by direct CD40-CD40L interactions between CD8 and CD4 T cells (15). In contrast, the primary CD8 T cell response against infectious pathogens was initially thought to be largely independent of CD4 T cell help (16–18). This is because pathogens can provide the inflammatory stimuli such as TLR ligands and induce the production of inflammatory cytokines required for full activation of APCs, and thus bypass the need for CD4 T cell help (18). However, recent studies have shown that primary CD8 T cell response to some pathogens, such as adenovirus (19), influenza virus (20), HSV-1 (21), and *Listeria monocytogenes* (22), is dependent on CD4 T cells. Thus, the nature of CD4 T cell help for primary CD8 T cell responses in the setting of infections remains to be defined.

Although the primary CD8 T cell response to infections can be independent of CD4 T cell help, recent studies have indicated that CD4 T cell help is required for the generation of long-lived, functional memory CD8 T cells that respond rapidly upon secondary exposure to pathogens (23–25). However, it remains controversial with regard to when the CD4 T cell help is needed for the generation of functional memory CD8 T cells. It has been suggested in some studies that CD4 T cell help during initial priming phase delivers the necessary "instructive" signals for the generation of a fully functional memory $CD8^+$ T cell pool (15, 23, 24). In contrast, other studies have suggested that signals derived from CD4 T cells are required for regulating homeostasis of the memory CD8 T cells (26). Furthermore, other observations have shown that the requirement for CD4 T cell help in memory CD8 T cell maintenance and function might be pathogen specific (20, 22).

In this study, we sought to better understand when CD4 T cell help is required for the primary and memory CD8 T cell responses using a murine model of vaccinia virus (VV) infection. Here, we found that the clonal expansion of Ag-specific CD8 T cells was severely compromised in CD4-deficient ($CD4^{-/-}$) mice or wild-type (WT) mice depleted of CD4 T cells. The reduced clonal expansion of CD8 T cells was not caused by a defect in T cell activation or proliferation, but rather by poor survival of activated T cells, suggesting CD4 T cell help is crucial for the survival of CD8 T cells during the primary response. As a result, a much smaller,

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[3] Abbreviations used in this paper: DC, dendritic cell; VV, vaccinia virus; WT, wild type; HA, hemagglutinin; MFI, mean fluorescence intensity; AICD, activation-induced cell death; LCMV, lymphocytic choriomeningitis virus; LN, lymph node.

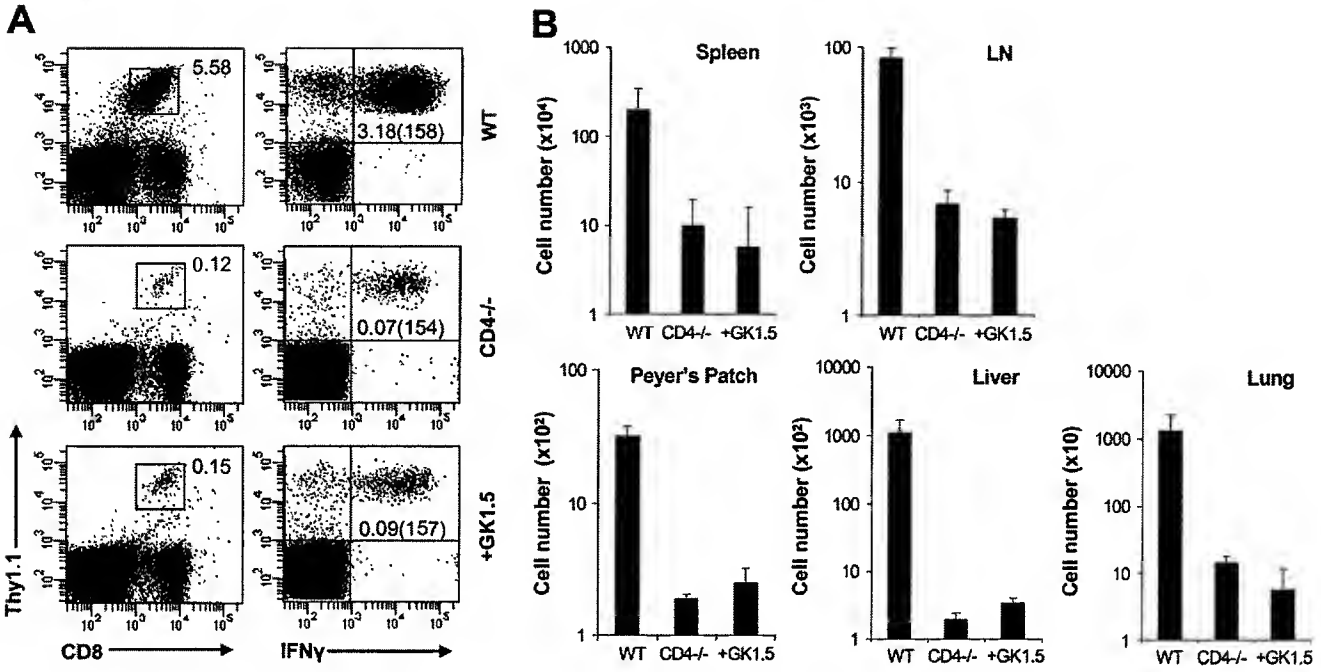

FIGURE 1. Defective clonal expansion of CD8 T cells in the absence of CD4 T cell help during a primary response to VV infection. A total of 10^4 purified naive clone 4 CD8 T cells (Thy1.1$^+$) were adoptively transferred into congenic WT, CD4-deficient (CD4$^{-/-}$) B10.D2 mice (Thy1.2$^+$), or WT mice treated with the depleting CD4 mAb GK1.5 (+GK1.5), which were subsequently infected with rVV-HA. Seven days later, spleen and other lymphoid and nonlymphoid organs were harvested for analysis of transferred cells. *A*, Expansion and function of clonotypic cells. Splenocytes were stained with anti-CD8, anti-Thy1.1, and anti-IFN-γ intracellularly. Percentage of total (*left panels*) and IFN-γ-producing (*right panels*) clonotypic cells among total lymphocytes is indicated with the numbers in parentheses showing the MFI ($\times 10^2$) of IFN-γ-producing clonotypic cells. *B*, The mean absolute numbers of clonotypic T cells per spleen, combined six peripheral LNs, combined six Peyer's patches, whole liver, or whole lung are indicated with SDs. Data shown are representative of three independent experiments.

but relatively stable CD8 memory pool was generated in the absence of CD4 T cells. Furthermore, we observed that in addition to CD4 T cell help provided during the primary response, the "help" provided following a secondary challenge was also required for the survival of memory CD8 T cells during the recall expansion. These results suggest that CD4 T cell help is crucial for multiple stages of CD8 T cell response to VV infection. As VV has been used widely as vaccine vehicles for infectious diseases and cancer, our findings may have important implications for the design of effective vaccine strategies.

Materials and Methods

Mice

B10.D2 mice were purchased from The Jackson Laboratory. CD4-deficient mice (CD4$^{-/-}$) on the C57BL/6 background were purchased from The Jackson Laboratory and backcrossed onto the B10.D2 genetic background for nine generations. The clone 4 hemagglutinin (HA)-TCR-transgenic mice that express a TCR recognizing a K^d-restricted HA epitope (^{518}IYSTVASSL^{526}) were provided by Dr. L. Sherman (The Scripps Research Institute, La Jolla, CA) (27). These mice were backcrossed for more than nine generations onto the Thy1.1, B10.D2 genetic background. All mice used for experiments were between 6 and 8 wk of age. All experimental procedures involving the use of mice were done in accordance with protocols approved by the Animal Care and Use Committee of the Duke University Medical Center.

Adoptive transfer of clone 4-transgenic T cells

Naive clonotypic HA-specific CD8$^+$ T cells (Thy1.1) were prepared from clone 4 TCR-transgenic mice. Briefly, single-cell suspensions were prepared from spleen and lymph nodes of clone 4 TCR mice and clonotypic percentage was then determined by flow cytometry analysis of CD8$^+$Vβ8.2$^+$ cells as described (28, 29). The activation marker CD44 was also checked to ensure these clonotypic cells were naive. CD8 T cells were positively selected using anti-CD8 microbeads according to the manufacturer's instructions (Miltenyi Biotec) with a purity of >98%. A total of 1×10^4 or 1×10^6 purified CD8$^+$ T cells were adoptively transferred to naive recipients via tail vein injection in 200 μl of HBSS. In some experiments, cells were labeled with CFSE before transfer as previously described (28).

Immunizations and Ab treatment

Recombinant vaccinia virus encoding HA (rVV-HA) and rE1-deleted adenovirus encoding HA (Ad-HA) were previously described (28). rVV-HA was grown in TK-143B cells, purified by sucrose banding, and titer was determined by plaque-forming assay on TK-143B cells. Mice were infected with 5×10^5 or 5×10^6 PFU rVV-HA i.p. Ad-HA was grown in 293 cells (American Type Culture Collection), purified by two rounds of CsCl density centrifugation, and desalted by gel filtration through Sephadex G-25 column (PD-10 column; Amersham Bioscience). The titer was determined by plaque-forming assay on 293 cells. Mice were infected with 2×10^9 PFU i.p.

In vivo CD4$^+$ T cell depletion in B10.D2 mice was performed by i.p. injection of the anti-CD4 mAb GK1.5 (150 μg) for 3 days beginning 10 days before rVV-HA infection and every third day thereafter until completion of the experiment as described (19).

Isolation of lymphocytes from nonlymphoid tissues

Lymphocytes were isolated nonlymphoid tissues as described (30). Briefly, liver or lung issue was homogenized and passed through a 70-μm cell strainer. The single-cell suspension was resuspended in 35 ml of HBSS and centrifuged on a 15 ml of Ficoll gradient (Amersham). Cells were harvested from the Ficoll gradient and washed twice with HBSS before analysis.

Abs and flow cytometry

mAbs (all from BD Biosciences unless indicated) used for staining were PE-Cy5-conjugated anti-CD8; FITC-conjugated anti-Thy1.1, -CD8, -CD44, -CD62L, -CD69, -IFN-γ, -CD122, -TNF-α, and -granzyme B (eBioscience); PE-conjugated anti-Tby1.1, annexin V, and anti-Bcl-x_L (Santa Cruz Biotechnology); biotin-conjugated anti-CD127. Collection of flow cytometry data was conducted using a FACScan or FACSCanto (BD

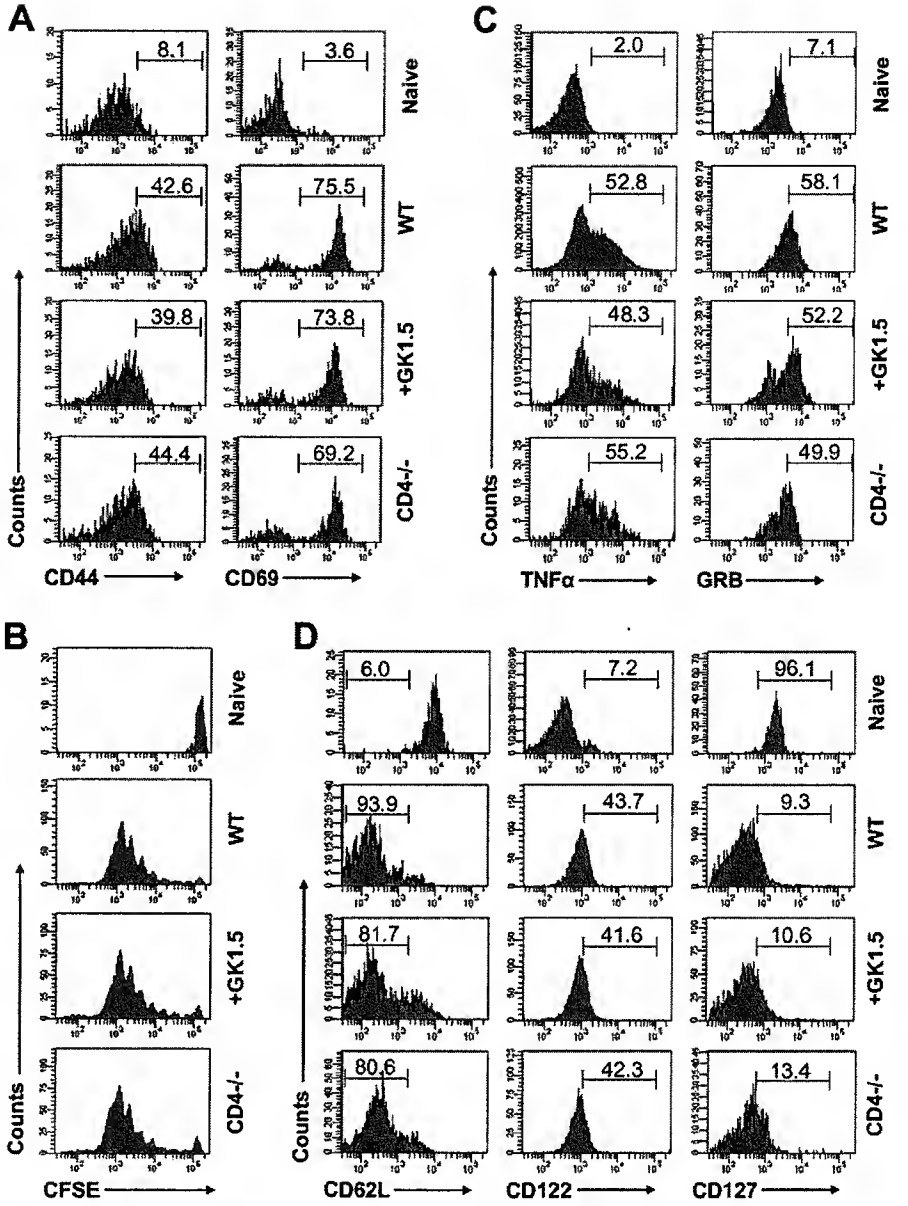

FIGURE 2. CD8 T cell activation and effector differentiation in response to VV infection is not affected by a lack of CD4 T cell help. *A* and *B*, A total of 10^6 purified naive clone 4 CD8 T cells were transferred to WT, $CD4^{-/-}$ mice, or WT mice treated with GK1.5 mAb (+GK1.5) that were subsequently infected with rVV-HA. Some WT mice were left uninfected (Naive). *A*, Twenty-four hours postinfection, splenocytes were harvested and stained with Abs against CD8, Thy1.1, and the activation markers CD44 or CD69. The percentages of $CD44^{high}$ and $CD69^{high}$ are indicated. *B*, Three days postinfection, in vivo division of CFSE-labeled clonotypic cells in the spleen was analyzed. *C* and *D*, A total of 10^4 purified naive clone 4 CD8 T cells were transferred into different recipients that were subsequently infected with rVV-HA. Seven days later, splenocytes were harvested, stained with anti-CD8 and anti-Thy1.1, and analyzed for the production of effector molecules and the expression of surface markers. *C*, The percentages of TNF-α and granzyme B (GRB) producing clonotypic cells are indicated. *D*, The percentages of $CD62L^{low}$, $CD122^{high}$, and $CD127^{high}$ are indicated. All plots are gated on $CD8^+Thy1.1^+$ cells. Data shown are representative of three independent experiments.

Biosciences) and events were analyzed using CellQuest or FACSDiva software (BD Biosciences).

Intracellular staining

To measure intracellular levels of Bcl-x_L, splenocytes were stained with anti-CD8 and -Thy1.1 Abs. Cells were then permeabilized using the Cytofix/Cytoperm kit according to the manufacturer's instructions (BD Biosciences) and subsequently stained intracellularly with anti-Bcl-x_L Ab. To assess production of effector molecules, splenocytes were cultured in 200 μl of CTL medium (RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 IU/ml streptomycin, and 50 μM 2-ME) at a concentration of 10^7 cells/ml in the presence of 2 μg/ml of the K^d HA518–526 peptide and 5 μg/ml brefeldin A containing Golgi-Plug (BD Biosciences) for 6 h at 37°C. After incubation, cells were washed and stained with anti-CD8 and -Thy1.1. Cells were then permeabilized using the same protocol as for Bcl-x_L and subsequently stained intracellularly with anti-IFN-γ, -TNF-α, or -granzyme B.

Real-time quantitative PCR

Total RNA was isolated from purified cells using TRIzol reagent (Invitrogen Life Technologies) and cDNA was generated using a reverse transcription kit (Promega). Real-time PCR was performed using an iCycler (Bio-Rad) to measure SYBR green incorporation. The following primer sets were used: Bcl-x_L, 5′-TGGTGGTCGACTTTCTCTCC-3′, 5′-CTCCA TCCCGAAAGAGTTCA-3′; TRAIL, 5′-TCACCAACGAGATGAAGC AG-3′, 5′-GGCCTAAGGTCTTTCCATCC-3′. Amounts of mRNA were normalized to hypoxanthine phosphoribosyltransferase RNA levels within each sample.

Memory T cell isolation and secondary transfer

Purified clone 4 CD8 T cells were adoptively transferred into naive mice as described above. Forty-five days, post-rVV-HA infection, mice were sacrificed and spleen, superficial lymph nodes, and mesenteric lymph nodes were pooled. Cells were stained with PE-conjugated anti-Thy1.1 and FITC-conjugated anti-CD8. $Thy1.1^+$ T cells were positively selected using anti-PE beads according to the manufacturer's instructions (Miltenyi Biotec). Enriched $Thy1.1^+$ cells were then subjected to cell sorting gated on $Thy1.1^+CD8^+$ with a high speed cell sorter FACSVantage (BD Biosciences). The purity of FACS-sorted populations of cells was >95%.

Ovary VV titer assay

Viral load in the ovaries was measured by plaque-forming assay as previously described (31). A total of 10^4 purified clone 4 CD8 T cells were transferred into female mice that were subsequently infected with 5×10^5 PFU rVV-HA. Mice were sacrificed 3 or 28 days postinfection and ovaries were harvested and stored at −80°C. Ovaries from individual mice were

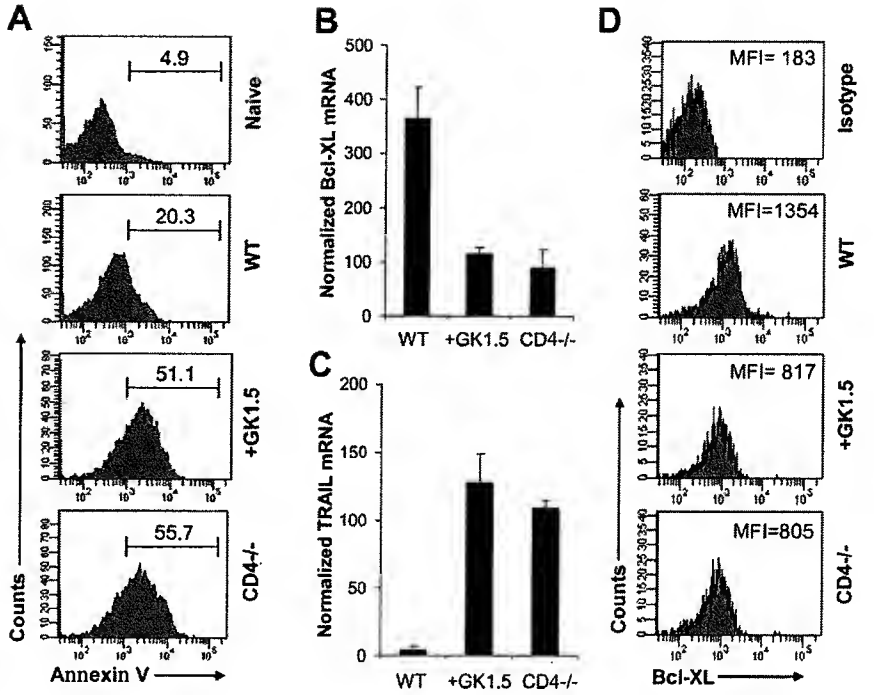

FIGURE 3. CD4 T cells promote the survival of activated CD8 T cells during priming in vivo. A total of 10^4 purified naive clone 4 CD8 T cells were transferred to WT, GK1.5-treated WT (+GK1.5), or $CD4^{-/-}$ mice that were subsequently infected with rVV-HA. Some WT mice were left uninfected (Naive). Seven days later, splenocytes were harvested for subsequent analysis. *A*, Annexin V staining. The percentage of Annexin V^+ cells among clonotypic cells is indicated. *B* and *C*, Clonotypic cells were purified by cell sorting and subjected to real-time quantitative PCR to measure the expression of Bcl-x_L (*B*) and TRAIL (*C*). Data are presented as normalized mRNA abundance to hypoxanthine phosphoribosyltransferase. *D*, Cells from different recipients were stained with Bcl-x_L intracellularly or an isotype control Ab (Isotype). The MFI of clonotypic cells is indicated. Plots are gated on $CD8^+Thy1.1^+$ cells. Data shown are representative of three independent experiments.

homogenized and freeze-thawed three times. Serial dilutions were performed and the viral titers were determined by plaque assay on confluent TK-143B cells.

Statistical analysis

Results were expressed as mean ± SD. Differences between groups were examined for statistical significance using the Student *t* test.

Results

CD4 T cell help is needed for clonal expansion of CD8 T cells during the primary response to VV infection

To better understand the role of CD4 T cells in primary and secondary CD8 T cell responses to infection, we used a model of influenza HA-specific CD8 T cell response to rVV-HA in vivo. A total of 10^4 naive clone 4 HA-specific CD8 T cells ($Thy1.1^+$) purified from clone 4 HA-TCR transgenic mice that express a TCR recognizing a K^d-restricted HA epitope, were transferred into either WT or CD4-deficient ($CD4^{-/-}$) B10.D2 mice ($Thy1.2^+$) that were subsequently infected with 5×10^5 PFU rVV-HA i.p. Seven days after infection, splenocytes were analyzed for clonal expansion and effector differentiation of the clone 4 CD8 T cells. Massive clonal expansion and effector differentiation as measured by the production of IFN-γ were detected in WT mice (Fig. 1). By contrast, the extent of clonal expansion was significantly ($p < 0.001$) diminished when clone 4 CD8 T cells were transferred into $CD4^{-/-}$ mice (Fig. 1). A similar degree of reduction in clonal expansion was found in other lymphoid and nonlymphoid organs including peripheral lymph nodes (LN), Peyer's patch, liver, and lung (Fig. 1*B*).

A recent report has shown that in $CD4^{-/-}$ mice, the endogenous CD8 T cell population contains a large fraction of MHC class II-restricted cells (32). To ensure the results seen in Fig. 1 were not due to a difference in the endogenous T cell repertoire, we performed the experiments in WT mice depleted of CD4 T cells using the depleting anti-CD4 mAb, GK1.5 before adoptive transfer of clone 4 $CD8^+$ T cells. Again, 10^4 purified clone 4 CD8 T cells were adoptively transferred into either untreated (WT) or GK1.5-treated (+GK1.5) B10.D2 mice that were subsequently infected with 5×10^5 PFU rVV-HA. Seven days later, splenocytes were harvested for analysis. As in $CD4^{-/-}$ mice, clone 4 CD8 T cells in the CD4-depleted mice had a significant ($p < 0.001$) reduction in clonal expansion compared with the untreated WT mice (Fig. 1). Collectively, these results indicate that CD4 T cell help is critical for clonal expansion during the primary response to VV infection in vivo.

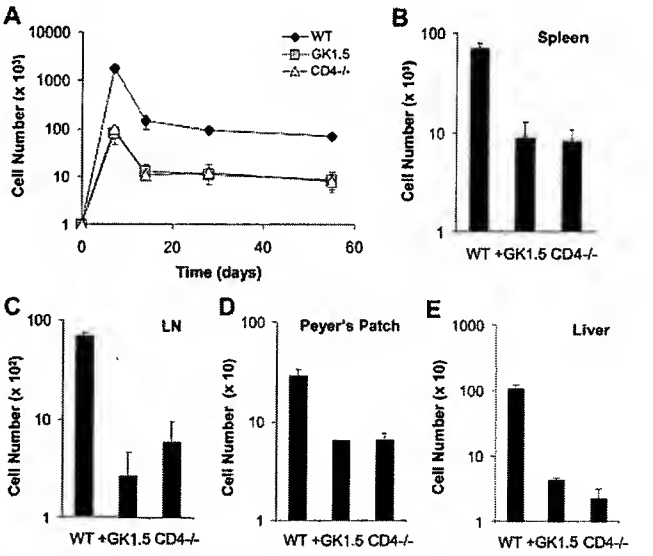

FIGURE 4. Diminished, but relatively stable, CD8 memory pool can develop in the absence of CD4 T cells. A total of 10^4 purified naive clone 4 CD8 T cells were transferred to WT, GK1.5-treated WT (+GK1.5), or $CD4^{-/-}$ mice that were subsequently infected with rVV-HA. *A*, Seven, 15, 28, and 55 days later, splenocytes were stained with anti-CD8 and -Thy1.1. The mean absolute numbers with SDs of clonotypic cells is indicated. *B–E*, Fifty-five days after infection, the mean absolute numbers with SDs of clonotypic cells per spleen (*B*), combined six peripheral LNs (*C*), combined six Peyer's patches (*D*), and whole liver (*E*), are indicated. Data are representative of two independent experiments.

Table I. *rVV-HA titer in ovaries at days 3 and 28 postinfection*[a]

	Day 3	Day 28
WT	$1.15 \times 10^3 \pm 212$	0
+GK1.5	$1.37 \times 10^3 \pm 495$	0
$CD4^{-/-}$	$1.20 \times 10^3 \pm 282$	0

[a] A total of 1×10^4 purified naive clone 4 CD8 T cells were transferred into WT, WT +GK1.5, or $CD4^{-/-}$ mice that were subsequently infected with 5×10^5 PFU rVV-HA. Three or 28 days later, ovaries were harvested and lysate was used to determine viral titer by plaque assay.

CD8 T cell activation and effector differentiation in response to VV infection is not affected by a lack of CD4 T cell help

We next investigated what contributed to the defect in CD8 T cell expansion during the primary response to VV infection in the absence of CD4 T cells. One possibility is that CD8 T cells are not fully activated without CD4 T cell help. To address this, we transferred 10^6 naive clone 4 CD8 T cells into WT, GK1.5-treated, or $CD4^{-/-}$ mice and subsequently infected the hosts with 5×10^6 PFU rVV-HA. Higher clone 4 T cell numbers (10^6) were used due to the fact that 10^4 transferred cells were below the limit of detection at early time points. Twenty-four hours after infection, clone 4 CD8 T cells in WT, GK1.5-treated, and $CD4^{-/-}$ mice displayed a similarly activated phenotype of $CD44^{high}$ and $CD69^{high}$ compared with the naive CD8 T cell phenotype of $CD44^{low}$ and $CD69^{low}$ (Fig. 2*A*). Three days after infection, CFSE-labeled clone 4 CD8 T cells in WT, GK1.5-treated, and $CD4^{-/-}$ mice underwent several rounds of division similarly by CFSE dilution (Fig. 2*B*), suggesting CD8 T cell proliferation was also not affected by a lack of CD4 T cell help. Furthermore, despite a reduced clonal size, the effector differentiation of clone 4 CD8 T cells in both GK1.5-treated and $CD4^{-/-}$ mice appeared to be intact at day 7 after infection as the production of IFN-γ on a per cell basis (as measured by mean fluorescence intensity (MFI)) was similar to that in WT mice (Fig. 1*A*). Similarly, the production of other effector molecules such as TNF-α and granzyme B appeared to be normal in GK1.5-treated and $CD4^{-/-}$ mice compared with that in WT mice (Fig. 2*C*). Additionally, the phenotype of effector CD8 T cells as measured by CD62L down-regulation, CD122 up-regulation, and CD127 re-up-regulation was not affected by the lack of CD4 T cells in both the GK1.5-treated and the $CD4^{-/-}$ hosts as compared with WT mice (Fig. 2*D*). These data suggest that CD8 T cell activation and effector differentiation in response to VV infection in vivo is not affected by a lack of CD4 T cell help.

The survival of activated CD8 T cells during priming is dependent on CD4 T cell help

Because CD8 T cell activation, proliferation, and effector differentiation do not appear to be altered due to the lack of CD4 help, we then asked whether the difference in clonal expansion could be due to decreased survival of the activated CD8 T cells in the absence of CD4 T cells. We used annexin V staining to assess CD8 T cells undergoing apoptosis. A total of 10^4 naive clone 4 CD8 T cells were transferred into WT, GK1.5-treated, or $CD4^{-/-}$ mice, followed by infection with 5×10^5 PFU rVV-HA. Seven days after infection, mice were harvested for analysis. Indeed, activated clone 4 CD8 T cells in both GK1.5-treated and $CD4^{-/-}$ mice displayed a significant ($p < 0.001$) increase in annexin V positivity (51.1 and 55.7%, respectively) compared with WT mice (20.3%, Fig. 3*A*). This increased apoptosis of activated CD8 T cells in the absence of CD4 T cell help correlated with a significant ($p < 0.001$) reduction in the expression of the prosurvival molecule, Bcl-x_L, at both the message RNA and protein levels (Fig. 3, *B* and *D*). TRAIL expression has been implicated in regulating secondary expansion of the "helpless" memory CD8 T cells (33). Here, we showed that TRAIL expression was also significantly ($p < 0.001$) up-regulated in the activated CD8 T cells during primary response to VV infection in the absence of CD4 T cells (Fig. 3*C*). Taken together, these results suggest that the diminished

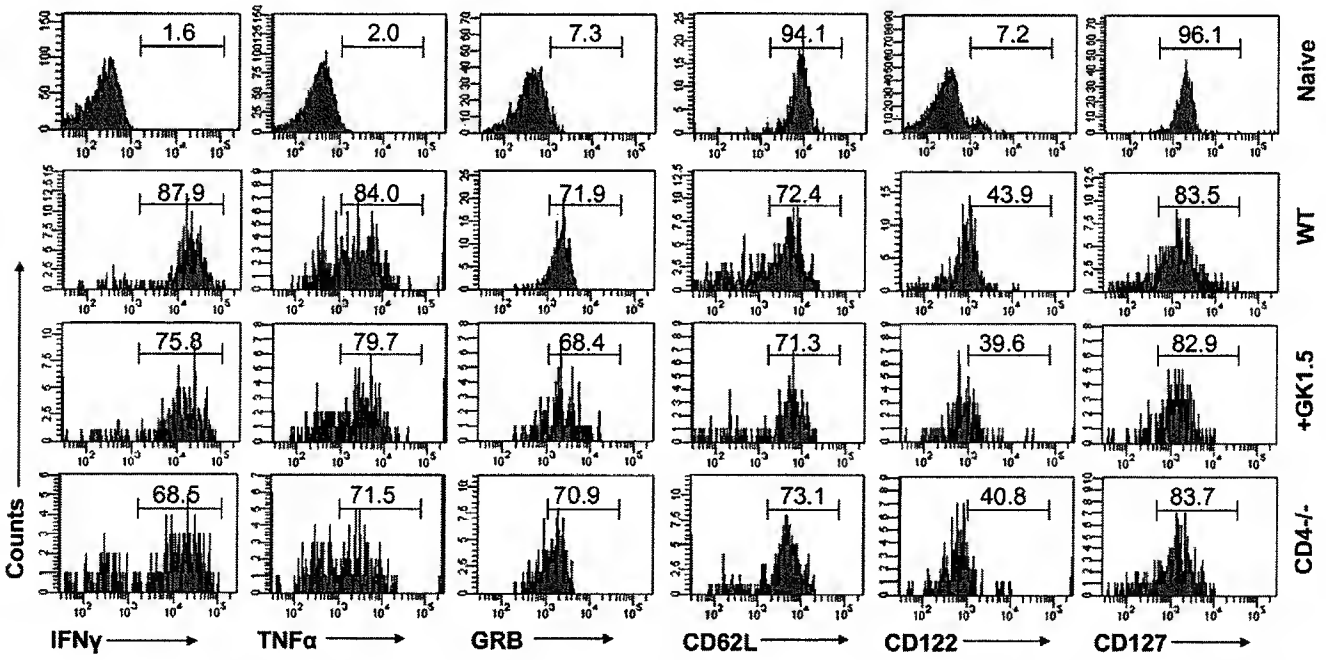

FIGURE 5. Phenotypic and function analyses of the "helpless" memory CD8 T cells. A total of 10^4 purified naive clone 4 CD8 T cells were transferred to WT, GK1.5-treated WT (+GK1.5), or $CD4^{-/-}$ mice that were subsequently infected with rVV-HA. Fifty-five days later, splenocytes were harvested for analyses. Percentages of IFN-γ^+, TNF-α^+, granzyme B^+, (GRB), $CD62L^{high}$, $CD122^{high}$, and $CD127^{high}$ are indicated. Plots are gated on $CD8^+Thy1.1^+$ cells. Data are representative of two independent experiments.

clonal expansion of CD8 T cells in response to VV infection in the absence of CD4 T cells is not caused by a reduction in T cell activation, but by poor survival of activated CD8 T cells.

Diminished, but relatively stable, CD8 memory pool can develop in absence of CD4 T cells

We next determined the ability of effector CD8 T cells to develop into stable memory cells in the absence of CD4 T cell help. After the peak of clonal expansion at day 7, splenic clone 4 effector CD8 T cells in the WT recipients underwent marked contraction between days 7 and 14, and those that survived developed into stable memory CD8 T cells (Fig. 4, *A* and *B*). This is consistent with previous observations in other models of bacterial or viral infections (1, 2, 6). Similarly, after contraction, clone 4 effector CD8 T cells generated in both the GK1.5-treated and $CD4^{-/-}$ hosts were also capable of differentiating into memory cells, but with a significant ($p < 0.001$) reduction in memory size that was proportional to the size of effectors (Fig. 4, *A* and *B*). This reduction was not a result of differential homing of memory cells in the absence of CD4 T help as a similar degree of decrease was observed in other lymphoid and nonlymphoid organs such as peripheral lymph nodes (Fig. 4*C*), Peyer's patch (Fig. 4*D*), and liver (Fig. 4*E*), in both the GK1.5-treated and $CD4^{-/-}$ mice. Neither was this decrease in the memory size due to a persistent viral infection, as viral titers performed on day 28 after infection showed that the virus was cleared in the GK1.5-treated and $CD4^{-/-}$ hosts as efficiently as WT mice (Table I). Despite a reduction in their size, the memory CD8 T cells generated in the GK1.5-treated and $CD4^{-/-}$ hosts appeared relatively stable at least up to day 55 after infection (Fig. 4*A*). Furthermore, the production of the effector molecules IFN-γ, TNF-α, and granzyme B, as well as the expression of the surface markers CD62L, CD122, and CD127 appeared to be similar in the WT, GK1.5-treated, and $CD4^{-/-}$ mice (Fig. 5). Thus, a diminished, but relatively stable memory CD8 pool can develop following VV infection in the absence of CD4 T cell help.

CD4 T cell help is also required for CD8 memory recall expansion following secondary challenge

One hallmark of memory cells is a rapid and more efficacious response upon secondary encounter with a pathogen. It is not entirely clear what controls this rapid recall potential of memory cells. Previous studies have suggested that CD4 T cell help during the primary response is needed for the generation of fully functional memory cells that can respond to secondary challenge rapidly (15, 23, 24). However, it is less clear whether CD4 T cells are needed following rechallenge for recall expansion. If so, what is the relative contribution of CD4 T cell help provided during the primary response vs following rechallenge to the recall expansion? To address these questions, clone 4 memory CD8 T cells were purified by FACS sorting from WT, GK1.5-treated, or $CD4^{-/-}$ mice 45 days after infection with rVV-HA. Equal numbers (3.5×10^4) of purified memory cells were then transferred into naive WT, GK1.5-treated, or $CD4^{-/-}$ mice that were subsequently challenged with 5×10^6 PFU rVV-HA i.p. Seven days after rechallenge, splenocytes were analyzed for the recall expansion and effector function of transferred memory cells. Vigorous recall expansion of clone 4 CD8 T cells was detected in WT recipients that received memory cells from WT donors, whereas the extent of recall expansion was significantly ($p < 0.05$) reduced in WT recipients transferred with memory cells from either GK1.5-treated or $CD4^{-/-}$ donors (Fig. 6). This is consistent with the notion that CD4 T cell help during the primary response provides the necessary "instructive" signals for the generation of fully functional memory cells (15, 23, 24). To our surprise, a much greater reduction in recall expansion ($p < 0.001$) was observed when memory cells from WT, GK1.5-treated, or $CD4^{-/-}$ donors were transferred into either CD4-depleted or $CD4^{-/-}$ recipients (Fig. 6). Similar results were obtained when mice were challenged with recombinant adenovirus-expressing HA (data not shown). These results indicate that CD4 T cell help provided following secondary challenge is also critical for recall expansion of memory CD8 T cells, in addition to that provided during the primary response.

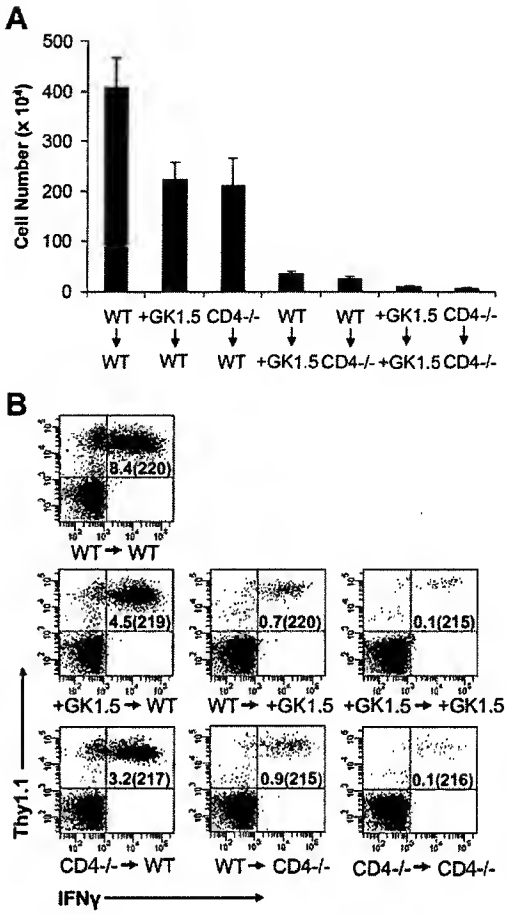

FIGURE 6. CD4 T cell help provided both during initial priming and following rechallenge is required for recall expansion of memory CD8 T cells. Purified naive clone 4 CD8 T cells were transferred to WT, GK1.5-treated WT (+GK1.5), or $CD4^{-/-}$mice that were subsequently infected with rVV-HA. Forty-five days later, clonotypic memory CD8 T cells were purified by FACS sorting and 3.5×10^4 memory cells from WT hosts were adoptively transferred into naive WT (WT → WT), GK1.5-treated (WT → +GK1.5) or $CD4^{-/-}$ (WT → $CD4^{-/-}$) recipients. Similarly, equal numbers of memory cells from either GK1.5-treated or $CD4^{-/-}$ hosts were transferred into WT (+GK1.5 → WT or $CD4^{-/-}$ → WT), GK1.5-treated (+GK1.5 → +GK1.5) or $CD4^{-/-}$ ($CD4^{-/-}$ → $CD4^{-/-}$) recipients. The recipients were subsequently challenged with rVV-HA. Seven days post-challenge, splenocytes were harvested and analyzed. *A*, The mean absolute numbers of clonotypic CD8 T cells with SDs are indicated. *B*, The percentages of IFN-γ-producing clonotypic CD8 T cells are indicated with the numbers in parentheses showing the MFI of IFN-γ-producing clonotypic CD8 T cells. Data shown are representative of three independent experiments.

CD4 T cell help promotes the survival of CD8 T cells during recall expansion

Despite the compromised recall expansion in the absence of CD4 T cell help either during the primary response or following rechallenge, the effector function of clone 4 CD8 T cells after recall

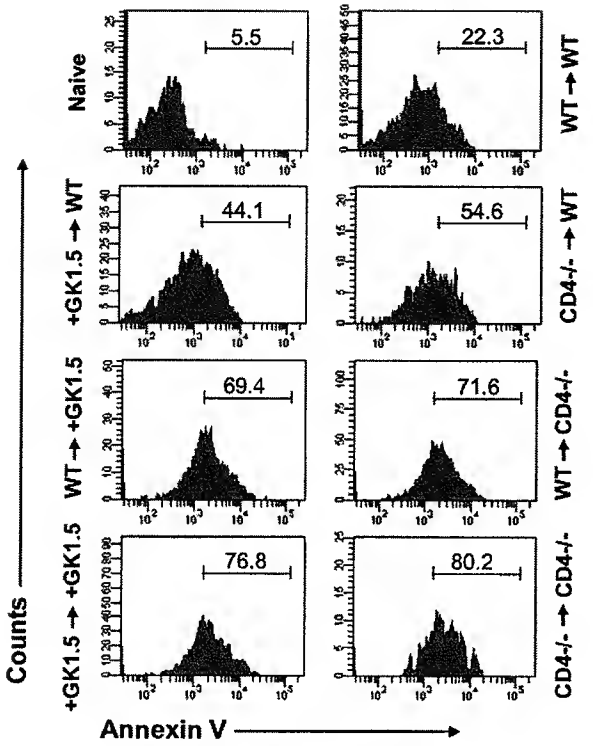

FIGURE 7. The survival of memory CD8 T cells during a recall expansion is dependent on CD4 T cells. A total of 3.5×10^4 memory cells were purified from day 45 rVV-HA-infected WT, GK1.5-treated (+GK1.5) WT, or $CD4^{-/-}$ mice and adoptively transferred into naive WT, GK1.5-treated (+GK1.5) WT, or $CD4^{-/-}$ recipients that were subsequently infected with rVV-HA. Seven days postchallenge, spleens were harvested and stained with anti-CD8, anti-Thy1.1, and annexin V. The percentage of Annexin V^+ cells among clonotypic cells is indicated. Plots are gated on $CD8^+Thy1.1^+$ cells. Data shown are representative of three independent experiments.

expansion appeared to be intact as their ability to produce IFN-γ on a per cell basis (as measured by MFI) was similar to that of "helped" WT control (Fig. 6*B*). This result suggested that the dependency of CD8 memory recall expansion on CD4 T cell help was likely mediated by promoting their survival, similar to our observations during the primary response. To address this question, clone 4 memory CD8 T cells (3.5×10^4) were purified from the WT, GK1.5-treated, or $CD4^{-/-}$ donors 45 days after infection with rVV-HA and transferred into WT, GK1.5-treated, or $CD4^{-/-}$ recipients that were subsequently infected with 5×10^6 PFU rVV-HA i.p. Seven days later, splenocytes were analyzed for clone 4 CD8 T cells that were undergoing apoptosis by annexin V staining. A significant ($p < 0.001$) increase in annexin V^+ cells was detected in WT recipients that received memory cells from either GK1.5-treated or $CD4^{-/-}$ donors (44.1 and 54.6%, respectively) compared with WT donors (22.3%, Fig. 7). A further increase in annexin V^+ cells was observed when memory cells from WT, GK1.5-treated, or $CD4^{-/-}$ donors were transferred into GK1.5-treated or $CD4^{-/-}$ recipients (Fig. 7). These results indicate that indeed CD4 T cell help during the primary and secondary response promotes the survival of CD8 T cells during recall expansion.

Discussion

In this study, we have shown that CD4 T cell help plays a critical role in both primary and memory CD8 T cell responses to VV infection. We demonstrate that although CD4 T cell help is not needed for activation and effector differentiation of Ag-specific CD8 T cells during a primary CD8 T cell response to VV infection, the survival of activated CD8 T cells is dependent on CD4 T cell help, leading to a reduction in clonal expansion and a diminished, but stable, CD8 memory pool. Furthermore, we also demonstrate that the "help" provided by CD4 T cells both during the primary response and following secondary challenge is required for recall expansion of memory CD8 T cells by promoting their survival.

It has been well-documented that CD4 T cell help is important for the induction of primary CD8 T cell response to noninflammatory Ags such as minor histocompatibility Ags, tumor Ags, or protein Ag in vivo (7–11). This is achieved by activating or "licensing" the DCs through CD40-CD40L interactions between DCs and CD4 T cells (12–14) or via direct CD40-CD40L interactions between CD8 and CD4 T cells (15). On the contrary, it had been initially thought that primary CD8 T cell response to infectious pathogens is largely independent of CD4 T cell help as pathogens can provide the inflammatory signals to promote full activation of DCs (16–18). However, some primary CD8 T cell responses to pathogens such as adenovirus (19), influenza virus (20), HSV-1 (21), and *L. monocytogenes* (22), are CD4 T cell help dependent. Because direct CD40-CD40L interaction between CD8 and CD4 T cells is not involved in these infections (34, 35), it has been unclear why CD4 T cell help is needed in these settings. Our results presented here demonstrate that CD4 T cell help is also required for primary CD8 T cell response to VV infection in vivo. Consistent with the notion that pathogens can activate DCs directly for efficient T cell priming and thus bypass the need for CD4 T cell help (18), the activation and effector differentiation of CD8 T cells during the primary response to VV infection is independent of CD4 T cells. However, the survival of activated CD8 T cells is critically dependent on CD4 T cell help and as a result, the clonal expansion of Ag-specific CD8 T cells is diminished without CD4 T cell help.

How does CD4 T cell help promote the survival of activated, Ag-specific CD8 T cells during the primary response in vivo? It is possible that CD4 T cells could either directly provide survival signals to activated CD8 T cells or indirectly act on an intermediate cell that provides CD8 T cells with such signals. A recent report in vitro has implicated CD4 T cells in protecting activated CD8 T cells from activation-induced cell death (AICD) through a direct cell-to-cell contact mechanism (36). Although AICD of CD4 T cells has been considered to be mediated by Fas-FasL interaction (37, 38), it remains controversial which death receptors are involved in AICD of CD8 T cells. Regulation of TRAIL expression by CD4 T cell help has been implicated in protecting memory CD8 T cells from AICD during a recall expansion (33). However, a recent study has suggested that CD4 T cell help consists of both TRAIL-dependent and -independent mechanisms (39). In line with these observations, we provided evidence that in the absence of CD4 T cell help, TRAIL expression is up-regulated in the activated CD8 T cells during the primary response to VV infection. In addition, there is a significant reduction in the expression of the pro-survival molecule, Bcl-x_L in the "helpless" CD8 T cells, suggesting that the intrinsic apoptotic pathway (40) may also be involved in CD4 T cell-mediated protection of activated CD8 T cells from AICD in vivo. Thus, future studies will be needed to elucidate the protective signals that CD4 T cells provide, and the signaling pathway(s) involved in promoting the survival of activated CD8 T cells during the primary response in vivo.

Despite the poor survival of activated CD8 T cells without CD4 T cell help during priming, which leads to a reduction in clonal expansion, these "helpless" effector CD8 T cells can develop into relatively stable memory cells albeit with a diminished memory size that is proportional to the size of effector T cells. This suggests

that after contraction phase, the maintenance of memory CD8 T cells after VV infection is independent of CD4 T cells. This is in contrast to the previous observation that the maintenance of memory CD8 T cells after an acute infection with lymphocytic choriomeningitis virus (LCMV) is compromised in MHC class II-deficient mice that lack CD4 T cells (26). The reasons for the discrepancy are not clear, but could be related to the pathogens used for the experiments. Indeed, recent studies have shown that the requirement for CD4 T cell help in memory CD8 T cell maintenance might be pathogen specific (20, 22). We have further observed that the "helpless" memory CD8 T cells are similar to the "helped" ones phenotypically as measured by the expression of CD62L, CD122, and CD127, as well as functionally in terms of the production of the effector molecules such as IFN-γ, TNF-α, and granzyme B. This is in contrast to a previous report with LCMV that the "helpless" memory CD8 T cells showed a $CD62L^{low}$ $CD122^{low}$ phenotype, suggesting a defect in the formation of $CD62L^{high}$ central memory cells (41). Again, it is not clear what contributes to the differences, but might be pathogen related.

The requirement for CD4 T cells in promoting fully functional memory CD8 T cells that can respond rapidly upon secondary challenge has been well-studied (15, 23–25). However, the majority of studies have focused on the CD4 T cell help provided during initial priming phase, which delivers the necessary "instructive" signals for the generation of fully functional memory $CD8^+$ T cells. It is less clear whether CD4 T cells are also needed following secondary challenge for the rapid recall expansion. Consistent with the previous observations, we have shown in this study that indeed CD4 T cell help is required during the primary response to VV infection for the generation of rapid recall response. We have also demonstrated that the presence of CD4 T cells following a secondary challenge is also crucial to the recall expansion of memory CD8 T cells. Our results are in contrast to the observations by Shedlock and Shen (24). In their study, only CD4 T cell help provided during initial priming with the VV-encoding gp33–41 epitope from LCMV was important for recall expansion following a secondary challenge. However, LCMV was used for the secondary challenge instead of VV. Because VV has been used extensively as vaccine vehicles for infectious diseases and cancer, our results may be more relevant to the design of effective vaccine strategies.

We have also provided evidence that defective recall expansion in the absence of CD4 T cell help either during the primary response or following rechallenge is due to poor survival. Similar to the requirement of CD4 T cells for the survival of CD8 T cells during the primary response, the mechanism(s) underlying the dependency of memory CD8 T cell survival on CD4 T cell help during recall expansion remains to be defined. Although TRAIL expression has been implicated in regulating the memory CD8 T cells that lack CD4 T cell help during initial priming from AICD during a recall expansion (33), it is not clear whether the same mechanism applies to the CD4 T cell help provided following secondary challenge. Future studies are needed to delineate the exact mechanism(s) by which CD4 T cells promote the survival of memory CD8 T cells during a recall expansion in vivo.

In summary, we have demonstrated that CD4 T cells are crucial to both primary and memory CD8 T cell responses to VV infection. This is achieved by promoting the survival of Ag-specific CD8 T cells during the initial priming and the recall expansion following rechallenge. As one major goal of vaccination is to maximize the magnitude of CD8 T cell response and to generate fully functional memory CD8 T cells, our results may have important implications for the design of effective strategies for treating infectious diseases and cancer.

Disclosures

The authors have no financial conflict of interest.

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ANNEX III

European Journal of Immunology

Immunoprevalence of the CD4$^+$ T-cell response to HIV Tat and Vpr proteins is provided by clustered and disperse epitopes, respectively

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Recent studies have suggested including nonstructural proteins as Tat and Vpr in HIV vaccines. However, little is known about the CD4$^+$ T-cell response that these small proteins induce in humans. We have therefore evaluated these responses by *in vitro* priming experiments of CD4$^+$ T lymphocytes harvested in healthy donors. In the Tat protein, only one peptide primed CD4$^+$ T cells of eight HLA unrelated healthy donors. T cells induced by this peptide recognized immature DC loaded with the native Tat protein and are restricted by multiple HLA-DR molecules, in agreement with its binding capacity. This peptide was therefore processed in an appropriate manner and was highly immunoprevalent. CD4$^+$ T-cell response to Vpr peptides was more disperse and involved six different peptides depending on the HLA-DR molecules of the donors. Two overlapping peptides were T-cell stimulating in at least half of the donors. T-cell response to Vpr in multiple donors is the result of a combination of several CD4$^+$ T-cell epitopes with good to moderate immunoprevalence. Altogether, our results show that the frequency of responders to HIV Tat or Vpr proteins relies on one or multiple CD4$^+$ T-cell epitopes, respectively.

Key words: CD4$^+$ T cells · Epitopes · HIV · HLA class II · MHC

Introduction

Twenty years after the discovery of HIV, an effective prophylactic or therapeutic vaccine is still not available. Current approaches are mainly based on the introduction of structural antigens into viral vectors, their combination with strong adjuvants or their injection as DNA vaccine [1]. Alternative or complementary approaches aim at neutralizing accessory or regulatory proteins because they disturb the immune system and counteract its beneficial role [2, 3]. Transacting protein (Tat) is a small regulatory protein that is expressed early in the viral cycle and is essential for viral replication [4]. Besides its transactivating activity, Tat exerts a wide range of activities. It diminishes T-cell function and provokes immunosuppression [5]. It enhances DC maturation [6] and exhibits an adjuvant activity [7]. Tat also regulates apoptosis of infected and noninfected cells by a variety of possible mechanisms including downregulation of Bcl-2 and upregulation of Bax and Caspase-8 [8]. In contrast to Tat, the Virus protein R (Vpr) accessory protein is expressed late in the virus cycle and is also essential for *in vivo* viral replication [9]. Vpr impairs DC maturation and T-cell activation [10]. Both Vpr and Tat proteins could be targeted by CD8$^+$ T-cells in seropositive donors [11–15] and hence constitute potential candidates to elicit an HIV-specific cellular response. Multiple forms of Tat peptides elicit neutralizing antibodies in monkeys and lead to promising but controversial results in terms of protection [16–18]. It is not known whether immune response specific for accessory or regulatory HIV proteins leads to immune escape,

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which has a fitness cost on the virus. Moreover, despite the requirement of $CD4^+$ T lymphocytes to sustain both humoral and cellular responses, the $CD4^+$ T-cell response specific for Tat and Vpr is poorly documented in humans [3, 19], in contrast to other HIV proteins [20–22].

Because of their small size, the $CD4^+$ T-cell response specific for Tat and Vpr proteins is expected to largely depend on the HLA molecules. As illustrated by pioneering studies on the genetic control of the immune response in mice [23] and humans [24], small proteins lead to high and low responders because of the presence or absence of peptide sequences able to be presented to T cells by the MHC class II molecules [25]. In humans, a high frequency of responders to a candidate vaccine is a requisite, but the underlying mechanisms of this process are poorly understood. Because confusion exists between immunodominance and frequency of responders, we use the term immunoprevalence to qualify the frequency of responders, while immunodominance is used to characterize the T-cell epitopes recognized in the context of priming with the native antigen [26, 27]. Because of the polymorphism of HLA II molecules, immunoprevalence of the T-cell epitopes is expected to rely on their capacity to bind to HLA II molecules. Peptides that are promiscuous for multiple HLA-DR molecules [20, 28, 29] or that bind to preponderant HLA II molecules as HLA-DP4 [30] generally exhibit a high immunoprevalence. Inter-individual variations in the T-cell response also result from the disparity of the Tcr repertoire [29, 30]. In order to predict and understand the human T-cell response to HIV Tat and Vpr proteins, we have therefore evaluated the immunoprevalence of their $CD4^+$ T-cell epitopes in healthy donors.

Results

Multiple Tat and Vpr peptides bound to multiple HLA-DR molecules

To characterize the $CD4^+$ T-cell response to HIV Tat and Vpr proteins, we submitted overlapping peptides encompassing their sequences to HLA-DR-specific binding assays. Because of the requirement for an aliphatic or aromatic residue in the N-terminal part of the peptides to ensure their binding to HLA-DR molecules, we selected overlapping peptides with these residues in positions 1–5 to optimize the screening. As a result, the Vpr and Tat sequences were covered by 16 and 9 peptides, respectively. They overlapped by up to 12 residues and only excluded the 60–64 and 80–86 Tat sequences. The data were presented as relative affinities to easily compare their binding properties to high binder peptides that we used as references. In the Tat protein (Table 1), most of the binding activities were found for peptides of the central part of the protein, namely Tat 24–38, 30–44, 34–48 and 41–55. These peptides bound to 3–6 HLA-DR molecules. In the Vpr protein (Table 2), multiple peptides exhibited binding activity comparable to that of the reference peptides. Vpr peptides (Vpr 29–43, 32–46, 35–49, 48–64, 52–66, 65–79 and 70–84) were retained for further investigations because they bound to at least three molecules and encompassed different peptide regions. Selected Tat and Vpr peptides were then investigated for their capacity to prime *in vitro* a $CD4^+$ T-cell response.

In contrast to the Vpr protein, the T-cell response specific for the Tat protein is mainly focused on one peptide only

Immunogenicity of the selected peptides was investigated by primary stimulation assays as previously described [29]. Peptides were tested in two different pools for the Tat and Vpr proteins, respectively. $CD4^+$ T lymphocytes were collected from nine HIV seronegative donors, who exhibited a diversity of HLA-DR molecules, including all the HLA-DR molecules we used in the binding experiments (Tables 3 and 4). Purified $CD4^+$ T lymphocytes were seeded in 48–60 wells per donor and per peptide pool and stimulated by autologous peptide-loaded mature DC and a cocktail of cytokines. Rare peptide-specific T-cell precursors were amplified by three weekly stimulations and characterized by IFN-γ ELISPOT. One hundred and fifty one peptide-specific T-cell lines were identified. Eighty nine T-cell lines were specific for Tat peptides, while 62 were specific for Vpr peptides (Tables 3 and 4). Because of the initial distribution of the $CD4^+$ lymphocytes, most of the T-cell lines are specific for one peptide only as illustrated by T-cell lines #205.6, #205.20, #205.27, #237.39, #205.63 and #191.43 (Fig. 1) or are specific for two overlapping peptides (#205.65 and #191.55) (Fig. 1). This strongly suggests that they derived from one $CD4^+$ T-cell precursor only. Interestingly, although the donors greatly differed in their HLA-DR molecules, almost all the T-cell lines primed with the Tat peptide pool were specific for the Tat 41–55 peptide (Table 3). Half-maximal stimulation of the T-cell lines required a peptide concentration ranging from approximately 10^{-2} to 10^{-1} μg/mL (Fig. 1). Few T-cell lines were specific for other peptides (Tat 24–38 and 30–44) (Table 3) and appeared less efficient in recognizing them (Fig. 1, T-cell line #237.39). In contrast to the Tat protein, the Vpr protein exhibited multiple T-cell-stimulating peptides (Table 4). Six peptides were immunogenic with a responder frequency varying from 22 to 66%. The most frequent T-cell-stimulating peptides were Vpr 48–64 and Vpr 52–66. One donor was a nonresponder and two donors responded by one peptide only. The other donors responded by two to five different Vpr peptides. Three peptides in the Tat protein and six peptides in the Vpr protein were therefore immunogenic, the peptide Tat 41–55 being T-cell stimulating in almost all the donors.

The T-cell response to Tat and Vpr peptides is restricted to multiple HLA-DR molecules

To characterize the HLA II molecules involved in the Tat and Vpr peptide presentation, we used L cells transfected with HLA II molecules corresponding to the typing of the donor, as APC.

Table 1. HLA II binding activity of overlapping HIV Tat peptides[a)]

Peptides	Relative binding activity										Number of HLA II
	DR1	DR3	DR4	DR7	DR11	DR13	DR15	DRB3	DRB4	DRB5	
Tat 1–15	46057	>302	>3247	>14697	>6899	**20**	1656	>714	2912	>11242	1
Tat 6–20	27450	>302	>3247	>14697	184	**7**	288	>714	>4932	699	1
Tat 9–23	90909	>302	>3247	>14697	>6899	>492	>3731	>714	>4932	1854	0
Tat 24–38	>66135	**0.5**	>3247	96	>6899	**3**	87	65	53	>11242	6
Tat 30–44	195	>302	>3247	58	15	200	>3731	>714	>4932	**23**	3
Tat 34–48	471	**2**	>3247	**40**	8	>492	>3731	>714	159	170	3
Tat 41–55	54545	**48**	>3247	>14697	**83**	**0.5**	>3731	>714	>4932	**0.3**	4
Tat 45–59	12564	>302	>3247	6085	894	**1**	>3731	>714	>4932	3429	1
Tat 65–79	72727	>302	>3247	>14697	2280	>492	>3731	>714	>4932	>11242	0

[a)] Peptide binding capacity was investigated by competitive ELISA. Data were expressed as relative activity (ratio of the IC_{50} of the peptide to the IC_{50} of the reference peptide, which is a high binder to the HLA II molecule). Relative activities inferior to 100 are in bold and correspond to active peptides. Means were calculated from at least three independent experiments.

Table 2. HLA II binding activity of overlapping HIV Vpr peptides[a)]

Peptides	Relative binding activity										Number of HLA II
	DR1	DR3	DR4	DR7	DR11	DR13	DR15	DRB3	DRB4	DRB5	
Vpr 1–15	>66135	>302	>3247	>14697	>6899	>492	>3731	>714	>4932	>11242	0
Vpr 16–30	>66135	>302	>3247	>14697	138	>492	>3731	>714	>4932	>11242	0
Vpr 20–35	**22**	>302	>3247	>14697	76	>492	**2**	>714	>4932	>11242	3
Vpr 24–38	11572	>302	>3247	4320	**30**	**4**	447	45	>4932	1022	3
Vpr 29–43	389	>302	>3247	690	**21**	**2**	**20**	>714	286	**46**	4
Vpr 32–46	**2**	>302	100	**1**	**61**	**6**	**28**	717	349	176	5
Vpr 35–49	**0.3**	>302	122	**1**	439	**3**	**2**	609	382	**23**	5
Vpr 40–54	>66135	**13**	>3247	164	204	**62**	194	505	1080	>11242	2
Vpr 44–58	1697	>302	173	229	2171	>492	**6**	>714	>4932	3020	1
Vpr 48–64	735	**6**	1876	**74**	207	>492	493	>714	>4932	**2**	3
Vpr 52–66	151	**0.4**	100	**60**	**15**	**2**	**83**	>714	>4932	**57**	6
Vpr 58–72	>66135	**1**	>3247	191	>6899	**2**	302	>714	>4932	>11242	2
Vpr 61–75	2646	**0.3**	**76**	327	**93**	**1**	**107**	**118**	**70**	**93**	6
Vpr 65–79	**30**	**2**	144	105	**1**	**1**	**8**	157	**2**	**3**	7
Vpr 70–84	**21**	**33**	147	276	**35**	**0.4**	438	>714	**33**	**57**	6
Vpr 79–93	>66135	>302	>3247	>14697	**75**	**1**	>3731	>714	>4932	>11242	2

[a)] Peptide binding capacity was investigated by competitive ELISA. Data were expressed as relative activity (ratio of the IC_{50} of the peptide to the IC_{50} of the reference peptide, which is a high binder to the HLA II molecule). Relative activities inferior to 100 are in bold and correspond to active peptides. Means were calculated from at least three independent experiments.

Twenty-nine T-cell lines were included in this study. Representative data are presented in Fig. 2. $CD4^+$ T-cell lines specific for the peptide Tat 41–55 were at least restricted to DR11 and DRB5. Some T cells were stimulated in a promiscuous manner and could also recognize the peptide Tat 41–55 presented by DR3 and DR15. As illustrated by the T-cell line #205.6, stimulation also occurred with the $DR13^+$ EBV cell line HHKB. For the Vpr peptides, we observed that Vpr 35–49 and 48–64 were restricted to HLA-DRB5, Vpr 52–66 was restricted to DR11 and DRB5, Vpr 65–79 to DR11 and Vpr 70–84 to DR3. We therefore showed that presentation to T cells of the Vpr and Tat peptides involved multiple HLA-DR molecules.

Peptide-specific T-cell lines recognized DC loaded with either Vpr or Tat protein

To limit the risk of LPS contamination, Vpr and Tat proteins were chemically synthesized and not produced by recombinant technology. As shown in Fig. 3 (left panels), T-cell lines specific for the Tat

Table 3. $CD4^+$ T-cell response induced *in vitro* by Tat peptides[a)]

Donors	HLA-DR typing		Number of specific T-cell lines		
	DRB1	2nd DR	Tat 24–38	Tat 30–44	Tat 41–55
P185	0701, 0404	DRB4			2
P191	0101, 1302	DRB3			3
P196	1101, 1104	DRB3			1
P200	0301, 1501	DRB3, DRB5	6		7
P205	1301, 1501	DRB3, DRB5			17
P228	0701, 0901	DRB4			1
P237	0401, 0701	DRB4	2	4	
P236	1101, 1301	DRB3			5
P249	1501, 1301	DRB3, DRB5			47
Responder frequency			2/9 (22%)	1/9 (11%)	8/9 (89%)

[a)] $CD4^+$ T-cell lines from nine unrelated seronegative donors were obtained by weekly stimulation with autologous mature DC loaded with a cocktail of Tat peptides. Their peptide specificity was assessed by IFN-γ ELISPOT assays using autologous PBMC as APC. Peptides were used at 10 μg/mL in the ELISPOT assay.

Table 4. $CD4^+$ T-cell response induced *in vitro* by Vpr peptides[a)]

Donors	HLA-DR typing		Number of specific T-cell lines					
	DRB1	2nd DR	Vpr 32–46	Vpr 35–49	Vpr 48–64	Vpr 52–66	Vpr 65–79	Vpr 70–84
P185	0701, 0404	DRB4					1	
P191	0101, 1302	DRB3	2	2	1	1		2
P196	1101, 1104	DRB3				5	19	
P200	0301, 1501	DRB3, DRB5	2	2	1	1		1
P205	1301, 1501	DRB5, DRB3			2	2		6
P228	0701, 0901	DRB4			3			
P237	0401, 0701	DRB4						
P236	1101, 1301	DRB3				1		
P249	1501, 1301	DRB5, DRB3			15	2	4	
Responder frequency			2/9 (22%)	2/9 (22%)	5/9 (55%)	6/9 (66%)	3/9 (33%)	3/9 (33%)

[a)] $CD4^+$ T-cell lines from nine unrelated seronegative donors were obtained by weekly stimulation with autologous mature DC loaded with a cocktail of Vpr peptides. Their peptide specificity was assessed by IFN-γ ELISPOT assays using autologous PBMC as APC. Peptides were used at 10 μg/mL in the ELISPOT assay.

41–55, which were derived from two different donors (P200 and P205), were stimulated by immature DC loaded with the Tat protein, but not by DC loaded with Vpr. In contrast, the T-cell lines specific for the Vpr 48–64 and 52–66 peptides were stimulated by Vpr-loaded DC, but not by Tat-loaded DC. This was also true for the Vpr 65–79 peptide, but the Vpr presentation appeared less efficient. We could not, however, demonstrate presentation of the Tat protein to the Tat 30–44-specific T-cell line #237.39 (data not shown), but this T-cell line appeared barely able to recognize the peptide (Fig. 1). Lack of stimulation by Vpr-loaded DC was also observed for the Vpr 70–84 peptide, although the T-cell lines were sensitive enough to recognize low doses of peptides (data not shown). We therefore demonstrated that Tat 41–55, Vpr 48–64, Vpr 52–66 and Vpr 65–79 peptide-specific T-cell lines recognized the native antigens processed by the DC.

Peptide-specific T-cell lines can recognize other variants besides the LAI/IIIB sequence

Sequences of the peptide we used in the binding assays and T-cell priming experiments were retrieved from the LAI/IIIB isolate. Because of the wide sequence variation of HIV, we investigated whether the most frequent variants of the clade B and the consensus of other clades were antigenic for the peptide-specific T-cell lines. As shown in Fig. 4A, Tat 41–55 from the LAI/IIIB isolate (variant A) has a frequency of 20%. Almost all the T cells primed by this peptide recognized the most frequent variant (B) (Fig. 4B and C) and to a lesser extent the three other variants (C–E) (Fig. 4B and C). Vpr 52–66-specific T-cell lines exhibited different patterns of recognition of natural variants (Fig. 5A). Almost all of them were stimulated by variants B (Fig. 5B and C). A good cross-reactivity

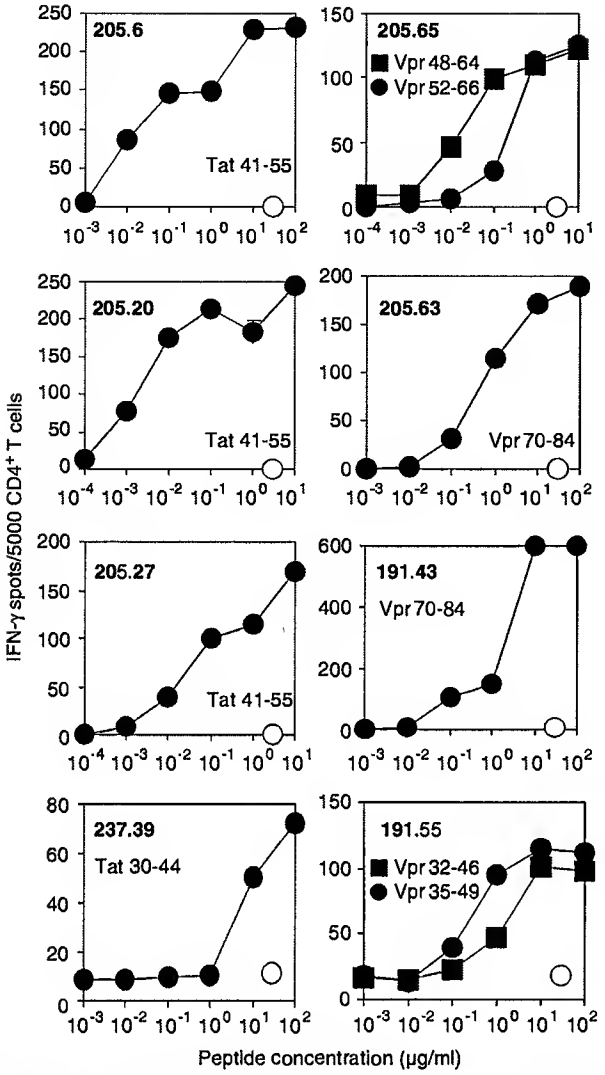

Figure 1. Peptide–dose response of T-cell lines induced by the selected Tat and Vpr peptides. Peptide-specific $CD4^+$ T-cell lines were obtained after weekly stimulations by autologous mature DC loaded with the Vpr or Tat peptide cocktail (10 μg/mL of each peptide). T-cell lines (5×10^3 cells/well) were analyzed by IFN-γ ELISPOT using autologous PBMC (5×10^4 to 1×10^5/well) as APC and at various peptide concentrations. Experiments were performed in duplicate. Each panel shows the result for one T-cell line. Each value of the peptide–dose responses is the mean of duplicate samples. Peptide specificity of each T-cell line was assessed in at least three independent experiments. Open circle: without peptide.

was shown for the T-cell lines #236.75 and #378.45, although most of the variants were not antigenic for the T-cell lines #236.66, #378.35 and #361.38 (Fig. 5B and C). Finally, a large cross-reactivity was observed for a Vpr 48–64-specific T-cell line, while it was very limited for a Vpr 65–79-specific T-cell line (data not shown).

Discussion

Although HIV Tat and Vpr are of interest for vaccine and protein engineering purposes, the $CD4^+$ T-cell response that can be raised in humans against these molecules remains largely unknown. We have therefore evaluated this response in healthy donors with the aim of evaluating the frequency of responders (immunoprevalence), characterizing their $CD4^+$ T-cell epitope content and identifying HLA-DR molecules that contribute to their immunogenicity. We observed that $CD4^+$ T-cells from all but one donor responded to Vpr peptides and all to Tat peptides. However, this frequent response is differently provided by their $CD4^+$ T-cell epitopes. It is mainly due to a single peptide in the Tat protein, while multiple peptides are responsible for the T-cell response in Vpr.

Our approach is based on the combination of assays of binding to preponderant HLA-DR molecules and of primary stimulation assays using $CD4^+$ T lymphocytes collected in seronegative donors. The use of binding assays to select promiscuous binders is consistent with many studies supporting the role of good affinity in the T-cell-stimulating properties of peptides [20, 28, 29]. *In vitro* priming experiments appear suitable to evaluate peptide immunogenicity because it reproduces *in vitro* the T-cell repertoire conditions encountered *in vivo* upon injection of prophylactic vaccines. It has been successfully used to identify T-cell epitopes from tumor [28], HCV [29] and HIV antigens [21, 30]. T-cell-stimulating peptides identified by this approach have been found to be immunogenic in vaccination trials of naïve individuals [31].

In the Tat protein, we showed that the peptide-specific T-cell response was mainly focused on one $CD4^+$ T-cell peptide only. Tat 41–55 induced multiple $CD4^+$ T-cell lines in HLA unrelated donors and hence exhibited a high immunoprevalence. These T-cell lines were stimulated by DC loaded with the native protein, demonstrating that Tat 41–55 is appropriately processed by the DC. In contrast, Tat 30–44 did not seem to correspond to a naturally processed peptide, although it was immunogenic for two healthy donors. Both Tat 24–38 and Tat 30–44 appeared weakly immunogenic on the basis of the number of T-cell lines induced and the frequency of responders, although they bound to multiple HLA-DR molecules. This is reminiscent of our previous reports on HCV peptides [29] and HLA-DP4-restricted peptides [30]. We previously suggested that promiscuous or HLA-DP4-restricted peptides could be weakly immunogenic due to inter-individual variation of the naïve T-cell repertoire [29, 30]. In contrast, some $CD4^+$ T-cell epitopes such as the Tat 41–55 or HCV NS3 1250–1264 [29, 32] exhibit a high prevalence not only because they bind to multiple HLA class II molecules but also because they possess a specific repertoire in almost all the individuals. Accordingly, the influence of the size of the naïve T-cell repertoire on the intensity of the primary $CD4^+$ T-cell response has been recently highlighted in the mouse [33]. In agreement with the binding data, we demonstrated, using L cells transfected by a unique HLA-DR molecule, that the Tat 41–55-specific T-cell response was restricted to the DR11, DRB5 and DR3 alleles. Based on the data obtained using EBV cell lines with T-cell line #205.6, it was probably also restricted to DR13. Restriction by the DR15 molecules, which was observed for few T-cell lines, was more suspicious as we did not detect any binding to the immunopurified molecules. The corresponding T-cell lines were

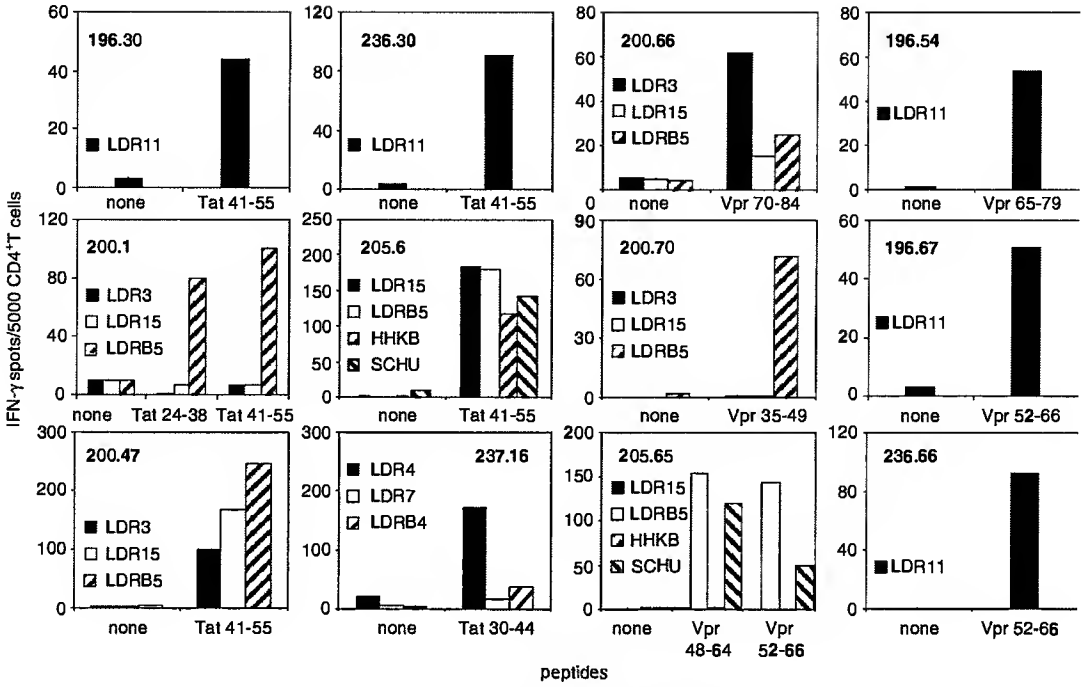

Figure 2. Restriction of the Vpr and Tat-specific T-cell lines. Peptide-specific $CD4^+$ T-cell lines were incubated with their appropriate peptide and L cells (3×10^4 cells/well) transfected by one HLA-DR molecule (LDR3, LDR4, LDR7, LDR11, LDR15, LDRB4 or LDRB5) and analyzed by IFN-γ ELISPOT. Homozygous EBV cell lines SCHU (DR1501, DRB5) and HHKB (DR1301, DRB3) were also used as APC for two T-cell lines. Each value represents the mean spot number of duplicates. Peptide specificity of each T-cell line was assessed in at least three independent experiments.

studied at high peptide concentration (10 μg/mL) only and could result from the T-cell priming by the DRB5/Tat 41–55 complex. Based on these data, it is expected that HLA-DR11, DR3, DR13 and DR15/DRB5 individuals are responders to Tat immunization and represent approximately 57% of the Caucasian population. In one study, approximately one third of seropositive patients exhibited a Tat-specific $CD4^+$ T-cell response, but the fine specificity of this response was not investigated [34]. Interestingly, Tat 41–55 overlaps the 31–50 [11] and 39–55 regions [12] and is close to the 30–37 sequence [15]. These regions are targeted by $CD8^+$ T-cells in multiple donors [11, 12, 15]. We therefore suggested that the long fragment 30–55 could elicit both $CD4^+$ and $CD8^+$ responses and hence constitute a potential candidate for epitope-based vaccine. As it also overlaps with the B-cell epitope 41–61, the 41–61 is expected to elicit a Tat-specific humoral response in humans [18]. Finally, its location, close to the protein transduction domain (Tat 49–57) could also participate in the design of Tat toxoids or peptides. Its preservation could enhance its own adjuvant activity [7] or the immunogenicity of engineered Tat [35]. Its disruption is expected to limit the immunogenicity of protein transduction domain fused proteins, which are targeted to the cytosol for nonimmunological purposes [36].

The Vpr protein exhibited a more complex content of $CD4^+$ T-cell epitopes than the Tat protein. Six peptides were immunogenic in the cellular assays. Two peptides, namely Vpr 48–64 and 52–66, primed $CD4^+$ T-cell lines in five and six out of nine individuals, respectively. None of these sequences have yet been reported. Only peptides 52–66 and 65–79 overlap the $CD4^+$ T-cell epitopes identified recently in the clade B consensus sequences [37]. In complete agreement with the binding data, we observed that Vpr 35–49 T-cell response could be restricted to the DRB5 allele, Vpr 48–64 to DRB5, Vpr 52–66 to DR11 and DRB5, Vpr 65–79 to DR11, and Vpr 70–84 to DR3, DR11 and DRB5. This is, however, a minimal list of restriction elements as these peptides activated specific T cells in healthy donors, harboring different HLA-DR molecules. Donor #237 did not give rise to peptide-specific T-cell lines, although the donor's HLA-DR molecules (DRB1*0401, DRB1*0701 and DRB4) bound the Vpr 65–79, 70–84, 48–64 and 52–66. Because we derived six Tat-specific T-cell lines from this donor, the lack of response to the Vpr peptide appeared specific to the peptides and not to the donor. As previously proposed [29], the absence of response may result from a deficit of Vpr peptide-specific naïve T cells in this donor in agreement with observations made in mice [33]. Previous studies showed that Vpr was targeted by $CD8^+$ T-cells [13–15] in a significant portion of seropositive donors [13–15]. $CD8^+$ T-cell epitopes were found to be spread all along the protein sequence [13, 14]. It is of note that the dominant HLA-A2-restricted T-cell epitope Vpr 59–67 [13] strongly overlaps with the Vpr 52–66 peptide we identified in this paper. As Vpr protein appeared to contribute to the virulence of HIV [9], activation of Vpr-specific $CD4^+$ and $CD8^+$ T lymphocytes is expected to participate in the HIV-specific immunity.

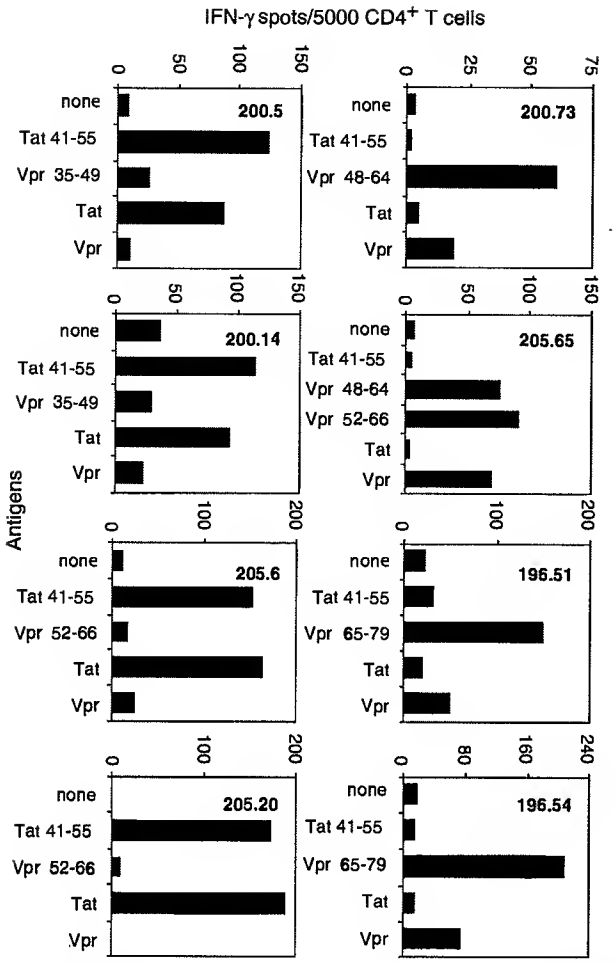

Figure 3. Presentation of the native proteins to the peptide-specific T-cell lines. T-cell lines (5×10^3 cells/well) induced by Tat peptides (left panels) or Vpr peptides (right panels) were incubated with immature DC (5×10^3/well) loaded with either the Vpr or Tat protein (3 μM) or the synthetic peptides (10 μg/mL). Their specificity was determined using IFN-γ ELISPOT assays. Experiments were performed in duplicate. Peptide specificity of each T-cell line was assessed in at least three independent experiments.

In conclusion, we have analyzed in detail the $CD4^+$ T-cell response specific to HIV Tat and Vpr proteins in humans, with a view to expediting the development of HIV vaccines.

Materials and methods

Peptides and proteins

Overlapping purified peptides (isolate LAI/IIIB) were provided by NeoMPS (Strasbourg, France). The 86-residue-long Tat protein (isolate LAI/IIIB) was synthesized using optimized Fmoc-chemistry protocols with a multichannel peptide synthesizer [38]. Vpr (isolate LAI/IIIB) was synthesized using similar protocols described previously [7]. After purification by reverse-phase HPLC, Tat and Vpr proteins were lyophilized and stored at −20°C in conditions preventing oxidation.

HLA II peptide-binding assays

HLA-DR molecules were purified from homologous EBV cell lines by affinity chromatography using the monomorphic mAb L243 [39, 40]. The binding to HLA-DR molecules was assessed by competitive ELISA as previously reported [39, 40]. Peptide concentration that prevented binding of 50% of the labeled peptide (IC_{50}) was evaluated. Data were expressed as relative affinity: ratio of the IC_{50} of the peptide to the IC_{50} of the reference peptide, which is a high binder to the HLA II molecule. Unlabeled forms of the biotinylated peptides were used as reference peptides to assess the validity of each experiment. Their sequences and IC_{50} values were the following: HA 306–318 (PKYVKQNTLKLAT) for DRB1*0101 (2 nM), DRB1*0401 (31 nM), DRB1*1101 (14 nM) and DRB5*0101 (8 nM), YKL (AAYAAAKAAALAA) for DRB1*0701 (7 nM), A3 152–166 (EAEQLRAYLDGTGVE) for DRB1*1501 (270 nM), MT 2–16 (AKTIAYDEEARRGLE) for DRB1*0301 (330 nM), B1 21–36 (TERVRLVTRHIYNREE) for DRB1*1301 (203 nM), LOL 191–210 (ESWGAVWRIDTPDKLTGPFT) for DRB3*0101 (9 nM) and E2/E168 (AGDLLAIETDKATI) for DRB4*0101 (20 nM).

Blood samples and HLA-DR genotyping

Blood cells were collected at the Etablissement Français du Sang (EFS, Rungis, France) as buffy-coat preparations from anonymous healthy donors after informed consent and following the guidelines of EFS. PBMC were isolated by density centrifugation on Ficoll-Hyperpaque gradients (Sigma Aldrich, St. Quentin Fallavier, France). HLA-DR genotyping was performed by using the Olerup SSP DRB1 typing kit (Olerup SSP AB, Saltsjobaden, Sweden).

Generation and specificity of antigen-specific T-cell lines

Immature and mature DC were generated from plastic-adherent PBMC by a five-day culture in AIM-V medium supplemented with 1000 U/mL of rh-GmCSF (R&D System, Lille, France) and 1000 U/mL of rh-IL-4 (R&D Systems, Abingdon, UK). LPS (Sigma) (1 μg/mL) was used as maturation agent. $CD4^+$ T lymphocytes were isolated by positive selection using an anti-CD4 MAB through an MACS, as recommended by the manufacturer (Miltenyi Biotech, Paris, France). Their purity was assessed by flow cytometry. They were diluted in IMDM (Invitrogen) supplemented with 24 mM glutamine, 55 mM asparagine, 150 mM arginine (all amino acids from Sigma), 50 U/mL of penicillin and 50 μg/mL of streptomycin (Invitrogen), and 10% human serum (hereafter referred to as complete IMDM). Mature

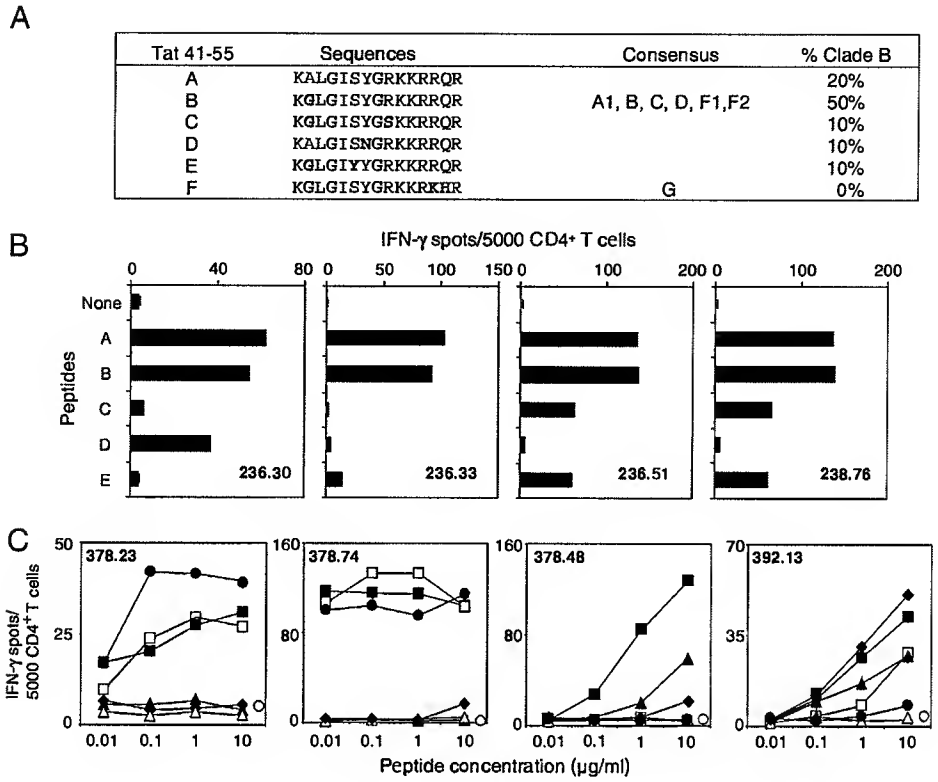

Figure 4. Recognition of Tat 41–55 variants by Tat 41–55-specific T-cell lines. (A) Sequence variation of HIV Tat 41–55 peptide. Consensus and frequency of the peptide sequence in the clade B the HIV sequences were from Los Alamos HIV databases (http://hiv-web.lanl.gov). (B and C) T-cell lines specific for the Tat peptide 41–55 were analyzed by IFN-γ ELISPOT using autologous PBMC (5×10^4 to 1×10^5 cells/well) and peptide variants using a single peptide concentration of 10 μg/mL (B) or a dose range of peptide (C). Each bar represents the mean spot number of duplicates. Peptide specificity of each T-cell line was assessed in at least three independent experiments. Closed square: variant A; open square: variant B; closed triangle: variant C; open triangle: variant D; closed diamond: variant E; closed circle: variant F; open circle: without peptide.

DC (5×10^5) were incubated at 37°C, 5% CO_2, for 4 h in 1 mL of complete IMDM containing a mixture of peptides, each peptide being at a concentration of 10 μg/mL. Pulsed cells were washed and added at 10^4 per round-bottom microwell to 10^5 autologous $CD4^+$ lymphocytes in 200 μL of complete IMDM with 1000 U/mL of IL-6 (R&D Systems) and 10 ng/mL of IL-12 (R&D Systems). Forty-eight to sixty wells per donor were seeded with the co-culture of DC and $CD4^+$ T lymphocytes. The $CD4^+$ T lymphocytes were restimulated on days 7, 14 and 21 with autologous DC freshly loaded with the HIV peptide mixture and were grown in complete IMDM supplemented with 10 U/mL of IL-2 and 5 ng/mL of IL-7 (R&D Systems). The stimulated $CD4^+$ T cells were investigated for their peptide specificity by IFN-γ ELISPOT assays at least 6 days after the last stimulation.

IFN-γ ELISPOT

Multiscreen HA plates (Millipore, St. Quentin en Yvelines, France) were coated with 2.5 μg/mL of mAb anti-human IFN-γ (1-D1 K, Mabtech, Stockholm, Sweden) in PBS (Invitrogen) for 1 h at 37°C and saturated with complete IMDM. APC were autologous PBMC (5×10^4 to 10^5/well), autologous immature DC (5×10^3/well) or HLA-DR-transfected L cells (3×10^4/well). HIV proteins were incubated for 4 h at 37°C at a concentration of 3 μM with immature DC that were subsequently washed before use. Peptides were directly added to the Multiscreen plates. $CD4^+$ T cells were seeded at 5×10^3/well. After overnight incubation at 37°C, captured IFN-γ was detected by subsequent addition of biotinylated mAb anti-hIFN-γ (7-B6-1; Mabtech) (0.25 μg/mL), extravidin-phosphatase (Sigma) and NBT/5-bromo-4-chloro-3-indolyl phosphate (Sigma). Spot number was automatically determined by the AID ELISPOT Reader System (AID, Strassberg, Germany). T-cell lines are considered as specific when their production of spots in the presence of antigens was at least two times higher than in their absence with a minimum of 20 spots per 5000 $CD4^+$ T cell.

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A

Vpr52-66	Sequences	Consensus	% Clade B
A	DTWAGVEAIIRILQQ	B, D	45.7%
B	DTWTGVEAIIRILQQ	C	8.6%
C	DTWAGVEALIRILQQ		4.7%
D	DTWAGVEAIIRTLQQ		4.3%
E	DTWAGVEAIIRMLQQ		3.4%
F	DTWAGVEAITRILQQ		2.6%
G	DTWAGVEAIIRVLQQ		2.2%
H	DTWTGVEALIRTLQQ		2.2%
I	DTWAGVEALIRILQQ	H	1.7%
J	DTWTGVEALIRILQQ		1.7%
K	DTWTGVEALIRMLQQ		1.3%
L	DTWVGVEAIIRILQQ		1.3%
M	DTWEGVEAIIRTLQQ	A, A1, A2	0%
N	DTWEGVEAIIRILQQ	F1, F2, G	0%

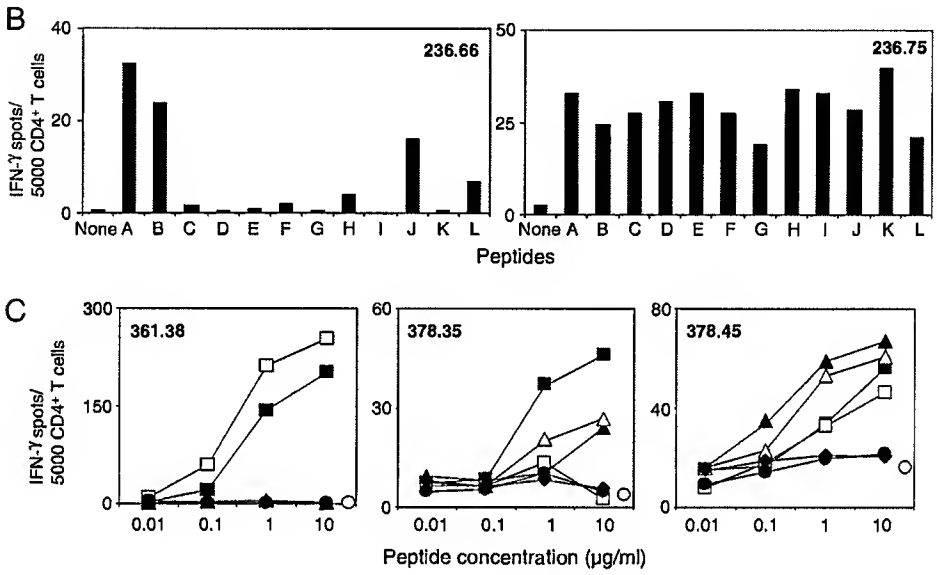

Figure 5. Recognition of Vpr 52–66 variants by Vpr 52–66-specific T-cell lines. (A) Sequence variation of HIV Vpr 52–66 peptide. Consensus and frequency of the peptide sequence in the clade B the HIV sequences were from Los Alamos HIV databases (http://hiv-web.lanl.gov). (B and C) T-cell lines specific for the Vpr peptide 52–66 were analyzed by IFN-γ ELISPOT using autologous PBMC (5×10^4 to 1×10^5 cells/well) and peptide variants using a single peptide concentration of 10 μg/mL (B) or a dose range of peptide (C). Each value represents the mean spot number of duplicates. Peptide specificity of each T-cell line was assessed in at least three independent experiments. Closed square: variant A; open square: variant B; closed triangle: variant C; open triangle: variant D; closed diamond: variant M; closed circle: variant N; open circle: without peptide.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: **Tat:** transacting protein · **Vpr:** virus protein R

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Annex IV

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Helper T-cell recognition of HIV-1 Tat synthetic peptides.

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Abstract

The regulatory proteins coded by the human immunodeficiency virus, (HIV)-1 genome are expressed by the infected cells before the initiation of the synthesis of structural proteins and thus immune response directed against these proteins could destroy infected cells before the release of infectious virions. The evaluation of T-lymphocyte responses toward Tat, one of the main HIV-1 regulatory proteins, is therefore of interest. We selected a group of HIV-infected patients with retained response to the recall antigen purified protein derivative and tested their CD4+ helper T-cell response toward recombinant Tat and toward 12 soluble synthetic partially overlapping 15-16-mer Tat peptides in a proliferation assay. Three peptides (amino acids 17-32, 33-48, and 65-80) were significantly recognized by the helper T-cells from infected individuals but not by the nine HIV-1-negative control persons. Nine of the 14 patients (64%) responded to at least one of these Tat peptides. Of the identified immunodominant peptides containing T-cell epitopes, one (aa 65-80) was recognized in association with human leukocyte antigens DR-2 allele, while the others appeared to be promiscuous and were equally recognized in association with several DR molecules. The identified immunogenic peptides were analyzed for the predicted presence of T-cell antigenic sites by several algorithms and positive correlation was detected for each peptide. Our results thus indicate that Tat protein can induce a cell-mediated immune response and identify three peptides containing T-cell epitopes that may be of importance in vaccine development.

Annex V

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Immunogenicity of protein therapeutics

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Protein therapeutics, such as monoclonal antibodies, enzymes and toxins, hold significant promise for improving human health. However, repeated administration of protein therapeutics, whether natural or recombinant, often leads to the induction of undesirable anti-drug antibodies (ADAs), which interfere with or neutralize the effect of the drug. Although an immune response to foreign proteins can be expected and is well understood, the basis for the development of responses to therapeutic autologous proteins is the subject of some debate. Inflammatory components of the drug delivery vehicle, T cell responses, T and B cell epitopes in the protein drug, and the associated B cell response are all targets for interventions aimed at reducing ADA responses. Here, we review some theories put forward to explain the immunogenicity of therapeutic proteins and describe some emerging protein-engineering approaches that might prevent the development of anti-drug antibodies.

Two broad categories of immune response to protein therapeutics

The number of therapeutic protein products available for use in clinical settings has dramatically increased in recent years. This category of biomedical products, also known as biological therapeutics, includes recombinant human cytokines (e.g. α and β interferon), cellular growth factors (e.g. GM-CSF), hormones (e.g. glucagon), neuromuscular antagonists (e.g. botulinum toxin), blood products (e.g. clotting factor VIII) and monoclonal antibodies. Although therapeutic proteins are generally considered safe and non-toxic, anti-therapeutic protein antibodies can develop during treatment. Anti-therapeutic protein antibodies [known as anti-drug antibodies (ADAs)] might neutralize or otherwise compromise the clinical effect of therapeutics and can also be associated with serious adverse events related to cross-reactivity with autologous proteins. Examples of ADAs include those to botulinum toxin (used to treat dystonia) and to clotting factor VIII (FVIII), a therapeutic for Hemophilia A [1,2]. Botulinum toxin is a foreign antigen, whereas therapeutic FVIII could appear foreign to the recipient because Hemophilia A is usually associated with deletion or modification of the FVIII gene. The generation of ADAs is not particularly surprising in these instances because it might be considered a vaccine-like reaction to foreign protein. Immune responses to vaccination are related to the number of drug doses administered, delivery route, and adjuvant. ADAs can also develop to recombinant autologous proteins, such as erythropoietin [3], which should not, in theory, breach tolerance [4]. The development of auto-antibodies in such cases might be owing to degradation or aggregation of the protein drug, or to the administration of the protein in the presence of a 'danger signal', such as a toll-like receptor (TLR) ligand. This will be covered in more detail later in the review.

As highlighted previously, immune responses to protein therapeutics might be categorized into one of two types of immune response: (i) activation of the classical immune system by foreign proteins, similar to the immune response against pathogens or vaccines; and (ii) breach of B and T cell tolerance to autologous proteins – a complicated series of immunological events that is not fully understood (Table 1). These two mechanisms overlap but are slightly different. The most easily explained mechanism is a 'vaccine-like' response to foreign proteins, involving T cells, B cells, and the innate immune system. By contrast, human immune responses to autologous proteins might also involve overcoming the regulation of adaptive immune responses by T regulatory cells (Treg).

Immunological mechanisms: B cell activation can be T cell-independent (Ti) or T cell-dependent (Td)

To identify effective interventions that might prevent the development of ADAs, it is worthwhile to review the sequence of immunological events leading to ADA secretion (summarized in Figure 1a).

The sequence of events that lead to B cell activation, and the resultant production of antibodies, can be divided into Ti and Td scenarios. Ti activation of B cells occurs when structural features of certain molecules, such as polymeric repeats, induce the 'signals' required to stimulate activation of a B cell subset, although the complement component C3d might also have a role when a T-dependent immune response cannot be activated, leading to the development of immune memory [5]. Although Ti activation is often cited as a source of antibodies to protein therapeutics, Ti activation of B cells generally does not lead to affinity maturation or to the generation of memory B cells. In general, Td activation of B cells results in a more robust antibody response, isotype switching, and the development

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Table 1. Parallels and differences between classical immune responses to exogenous antigens and autologous (therapeutic) proteins[a]

	Classical immune response	Immune response to recombinant autologous (therapeutic) proteins
Antibody	Present	Might be induced if the protein is altered, enabling recognition by B cell receptors (IgM, IgG)
T cell response	Present	Might be present under some circumstances
T regulatory cells	Absent	Might control anti-self response
Inflammation	Not absolutely necessary	Probably contributes to over-riding T regulatory cells and subsequent immunogenicity
Adjuvants	Increase likelihood of immunogenicity by stimulating innate immune response	Probably required to breach tolerance
Usual character of immune response	Effector or inflammatory immune response	Tolerance

[a]The two slightly different but overlapping mechanisms involved in the development of immunogenicity in response to the use of therapeutic proteins: (i) the classical immune response to foreign proteins, and (ii) incomplete tolerance to autologous proteins.

of B cell memory. The induction of IgG class ADAs (measurable in standard immunogenicity assays) generally implies that the therapeutic protein is a Td antigen that has led to isotype switching [6]. Because Td responses require T cell recognition of epitopes contained in the protein drug, binding of peptide epitopes [derived from internal processing by antigen-presenting cells (APCs)] to HLAs (human leukocyte antigens) MHC class II molecules, and recognition of the epitope–HLA complex by activated helper T cells can be assumed to occur. In the absence of signals provided by the cytokines released as a result of T cell interaction with APCs, the naïve B cell does not mature. Indeed, without T cell help, activated antigen-specific B cells might be rendered anergic or undergo apoptosis (Figure 1b). Therefore, T cell recognition of the peptide epitopes derived from the antigen can be considered a key determinant of Td antibody formation.

Tolerance and the regulation of the anti-self response

Tolerance to self-proteins is a basic immune system feature and the development of ADAs to recombinant autologous proteins can be regarded as a 'breach' of tolerance. Although auto-reactive B cells can be found in normal individuals, these cells are not usually stimulated to produce antibodies by the circulating levels of native proteins. There might be many parallels between the development of autoimmune disease and immunogenicity of therapeutic proteins, including the involvement of Treg. Reduction of Treg immune responses and induction of T-effector responses are probably significant contributors to ADAs because the link between T cell (and HLA-restricted) immune response and the development of auto-antibodies is well defined [7].

Extrinsic factors contributing to immunogenicity

Repeated dosing, routes of immunization, effective adjuvants, and other co-factors that are known to contribute to effective vaccination are best avoided with protein therapeutics. For example, reducing the total number of doses, minimizing aggregates, diminishing T cell activation, and avoiding delivery vehicles that have adjuvant effects, are rational strategies for reducing immunogenicity. These factors are discussed in greater detail in the following sections.

'Danger' signals

B cells might begin to produce antibodies in response to an auto-antigen if stimulated by a second signal from T helper cells, or by direct stimulation via TLR ligands, such as products of microbial degradation. TLR ligands, such as bacterial DNA (CpG) have recently been shown to stimulate B cell responses directly in the absence of T cells [8]. Thus, even low-level contamination of recombinant therapeutic protein with bacterial DNA, lipids or endotoxins might contribute to the development of unanticipated immune reactions to autologous proteins [9].

Arrays

One potent way to induce high levels of IgG independent of T cell help is to present the self antigen arrayed on viruses and virus-like particles (VLP). For example, self antigens conjugated with papilloma VLP evoked a strong antibody response to self-antigens [10,11]. Highly repetitive arrays present in a protein therapeutic might, therefore, induce Ti ADAs.

Aggregates

The role of aggregates in the development of immune responses to proteins is well-defined, particularly with respect to intravenous immunoglobulin (IVIG) and human growth hormone. Aggregates might engender an immune response by cross-linking the B cell receptor, which activates B cells to proliferate, targets protein to the lysosomal pathway and efficiently elicits T cell help [12]. In addition, aggregates of proteins can present epitopes in an array form, evoking T cell-independent antibody induction (see previous section). Furthermore, aggregates are readily taken up by APCs and might drive dendritic cell (DC) maturation, enhancing Td immune response.

Endocytosis of the target

Whether or not a therapeutic protein (or monoclonal antibody) is immunogenic might depend on whether the protein interfaces with the protein-processing machinery of APCs. Proteins that are internalized because their target is endocytosed; for example, anti-tumor necrosis factor (TNF) antibodies against surface TNF might be more immunogenic than proteins that are not endocytosed (for example, anti-TNF antibodies against soluble TNF).

Protein modification

Protein modification by sequence modification, denaturation or introduction of alternative glycosylation sites might render a protein enough unlike 'self' to induce a

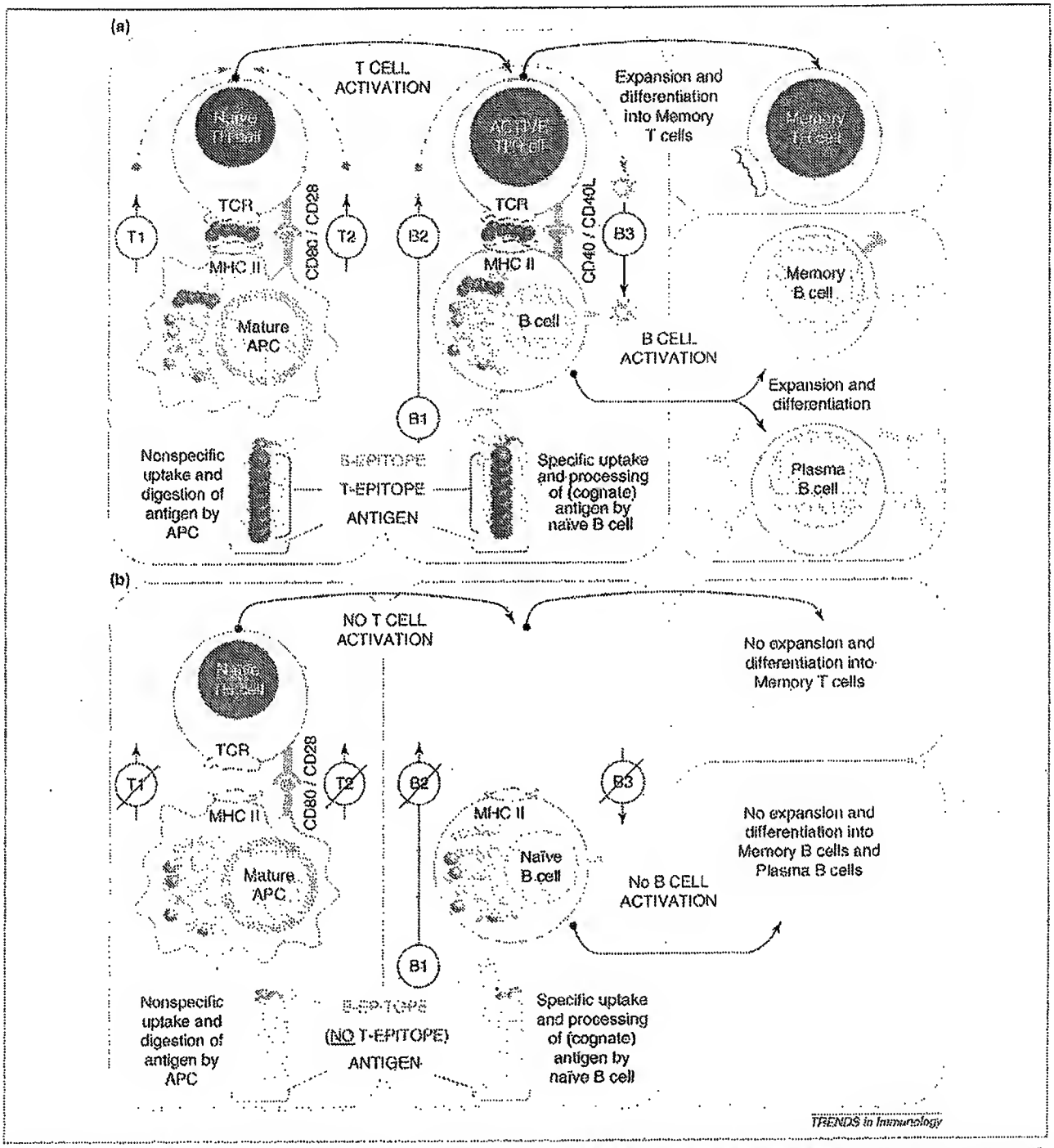

Figure 1. (a) To induce naïve B cells to produce antibodies to a protein antigen and to initiate an adaptive immune response, several events must be coordinated, usually within specialized regions of secondary lymphoid organs (e.g. lymph nodes, spleen, etc.). The first step in this process is the nonspecific internalization of the antigen by professional APC, such as mature DC. The mature DC process the antigen into peptides, which are then presented to naïve T cells in MHC class II molecules on the APC surface. The interaction of the TCR with this MHCII–peptide complex is indicated as T cell Signal 1 (T1). To fully activate the T cell, this must be accompanied by additional signals from what are termed 'co-stimulatory molecules', such as CD80 and CD86, provided by the APC, indicated as T cell Signal 2 (T2). In the absence of such a second signal, T cells might become anergic. Once fully activated, these CD4 T cells divide and can produce an array of cytokines with various activities. An interaction between B cell IgM and IgD receptors and cognate antigen is required to initiate activation of the naïve B cell (middle panel), this is termed B cell Signal 1 (B1). The B cell internalizes and processes the antigen–Ig complex and presents cognate T cell epitopes in the context of MHC class II on its surface. On encountering the cognate epitope–MHC complex, the T helper cell delivers cytokines, B cell Signal 2 (B2), which drive B cells to activate, proliferate, and differentiate into memory B cells and antibody-secreting plasma cells (right panel). Activated B cells express CD40 and bind CD40 ligand, communicating a further signal to the presenting B cell and the engaged T cell; T-B cell Signal 3 (B3). Thus, full activation of a B cell and its corresponding T cell is dependent on (i) proper presentation of the T cell epitope derived from the protein antigen by a professional APC and a B cell, and (ii) recognition of the peptide–MHC complex by the TCR. Cytokine signals from the T cell initiate a cascade of further immuno-stimulatory events that cause B cells to expand, switch isotypes and undergo phenotypic changes resulting in the establishment of memory B cells. (b) A complete lack of T cell epitopes would eliminate autologous T help and lead to B cell apoptosis.

classical immune response, while still resembling the human homolog enough to retain function. A single amino acid change or post-translational modification (or lack thereof) could create a neo-epitope for immune system recognition. Therapeutic proteins looking more like 'self', or with few mutations compared with the wild-type, are considerably less immunogenic than proteins that appear unique to the immune system. This is particularly true of mutated or fusion proteins containing novel antigenic B and T cell epitopes. In addition, proteins denatured during formulation might be more immunogenic than their intact counterparts [13]. These products might present new B cell or even T cell epitopes that were not present in the parent molecule, leading to the stimulation of an immune response [14].

Patient-dependent factors

Patients can show different types of immune response to therapeutic protein products, owing to allelic variation (e.g. allotypes), genetic deficiency, disease state (immunosuppressed or not) or HLA background. Allelic variation might have a more important role in the immunogenicity of autologous proteins than previously suspected and deserves greater study. For example, hemophilia patients with deletions or inversion of the FVIII gene have different immune responses to FVIII replacement therapy. Although the relationship between FVIII mutations and FVIII 'inhibitors' (neutralizing ADAs) needs to be better defined, two recent publications have documented the relationship between immunogenicity of selected proteins and HLA-associated T-dependent immune response [15,16]. Finally, some protein therapeutics might have immunological effects that contribute to, or are independent of, their own inherent immunogenicity (i.e. by perturbing key immune system components, such as anti-IL-2 or even IL-2 itself).

Leachates

Although the specific mechanism of action of soluble factors leached from uncoated rubber stoppers (so-called leachates) is unknown, they have been implicated in the formation of antibodies against recombinant erythropoietin (see following section). The leachates might have had a directly pro-inflammatory effect, similar to TLR ligands, providing the crucial 'danger signal' required for antibody induction.

Extrinsic factors that might diminish immunogenicity

Masking T cell epitopes with sugars (glycosylation), a process that occurs during the biosynthesis of the product, might reduce the immunogenicity of therapeutics. By contrast, addition of unusual sugars might increase immunogenicity [17,18]. Addition of polyethylene glycol (PEG) molecules (PEGylation) might camouflage proteins and interfere with ADA formation in addition to providing a means for prolonging the half-life of the product (see following section).

Consequences of antibodies to therapeutic proteins

Some types of antibodies do not interfere with the protein therapeutic, whereas others have dramatic and sometimes life-threatening effects. For example, antibodies that neutralize or interfere with the effect of the protein therapeutic and, occasionally, with the function of the endogenous analog, were initially described in the context of treatment with megakaryocyte growth and differentiation factor (MGDF) [4]. By contrast, severe general immune reactions, such as anaphylaxis associated with the use of animal antisera, have become rare, presumably because product purity has increased substantially over the past decades. Delayed-type infusion-like reactions resembling serum sickness are more common, particularly with monoclonal antibodies and other proteins administered in relatively large amounts. These reactions might be owing to immune complex formation. Patients with steadily increasing antibody titers are reported to show more infusion-like reactions than patients with short, temporary antibody responses. In many instances, the presence of ADAs is not associated with direct interference with protein function. Instead, the ADAs might influence the pharmacokinetics of the drug, potentially increasing or decreasing its half-life [19].

Neutralizing and cross-reactive ADAs

By contrast, neutralizing ADAs can contribute to loss of activity of the protein therapeutic. By definition, neutralizing antibodies that interact with a ligand–receptor interaction inhibit the efficacy of all ligands in the same class. For example, antibodies that neutralize recombinant erythropoietin (rEPO) by interfering with the binding of the protein to its receptor might interfere with all forms of erythropoietin, leading to anemia. Neutralizing antibodies are of greatest concern when they develop in conjunction with conditions for which there are no treatment alternatives [20]. Cross-reactive ADAs might also cause particular problems. In general, when neutralizing antibodies cross-react with an endogenous factor that has an essential biological function, there is a higher likelihood of adverse events. For example, ADAs were recently associated with the development of pure red cell aplasia (PRCA) following treatment with rEPO. This severe form of anemia was due to the development of antibodies cross-reactive with endogenous erythropoietin [21]. Cases of PRCA were associated with exposure to just one recombinant EPO product, suggesting that factors extrinsic to the protein itself were associated with the adverse outcome. Current evidence suggests that this effect was associated with a specific reformulation [22–24]. This formulation had replaced human serum albumin with polysorbate-80 and used uncoated stoppers in the product packaging. PRCA was 17 times more common in subjects exposed to this formulation than in those who were not. Cases of rEPO-associated PRCA dropped dramatically after the drug was reformulated and uncoated stoppers were replaced with coated versions [24].

Methods for the precise measurement of ADAs and their effects are essential for the rational design of future therapeutics. These methods have evolved rapidly over the past few years and have become the subject of two industry 'white papers'. Assays for measuring immune responses to therapeutic proteins are discussed in Boxes 1 and 2; see also the review by Sluis *et al.* [25]. Currently, the

Box 1. Pros and cons of assays for measuring immunogenicity: antibody assays

Several excellent reviews have been published regarding the development and validation of different assay formats for antibodies to therapeutic proteins [25]. Here we only discuss some caveats concerning interpretation of these means of measuring antibody response in *in vitro* assays.

Lack of standardization. Given that there are no international antibody assay standards, antibody titers measured in clinical trials are difficult to compare, unless the antibody assays were performed in the same laboratory. There is also wide variation in the reported incidence of immunogenicity associated with a single product. Lack of standardization is one explanation for these results.

Methodologies. Two different methodologies are used to evaluate patient serum for the presence of ADA. Methods evaluating antibody–drug binding include enzyme-linked immunosorbent assays (EIA), radioimmunoassay (RIA), and surface plasmon resonance (SPR). Binding assays and bioassays are often used in combination. For example, a sensitive binding assay might be used initially to screen samples for antibody and bioassays typically follow, because these are usually technically difficult and time-consuming.

Biological assays. Bioassays might be particularly useful for demonstrating that neutralization is caused by antibodies, if the presence of other inhibitory factors, such a soluble receptor, must be excluded.

Isotype. SPR technology can further characterize a neutralizing antibody response for isotype, affinity and specificity; however neutralizing capability cannot be linked to isotype.

Chronology. The immune response is a dynamic process and, therefore, the course of antibody development (kinetics) must be evaluated as the response evolves over time. Increasing titers of neutralizing antibodies are thought to be associated with epitope spreading and might correlate with some biological effect, however a particular therapeutic product might not be immunogenic enough to achieve such levels.

Measuring response in populations. An additional method for comparing relative immunogenicity in patient groups is to use 'mean population antibody titers' (describing the method by which the titer was determined). This is a more accurate means of describing immune responses to protein therapeutics than describing the percentage of subjects who convert from antibody-negative to antibody-positive.

Box 2. Pros and cons of assays for measuring immunogenicity: T cell immunogenicity monitoring

Monitoring T cell responses *in vitro* requires measurement of an indicator (usually a cytokine, or a T cell function, such as proliferation), in the presence of antigen.

Methodologies. Several different assays can be used to measure T cell responses. The assays measure proliferation of T cells in response to an antigen (thymidine incorporation) or release of cytokines, such as IL-2 or IFNγ, by T cells responding to antigen (ELISA or ELISpot assays). In every case, T cell responses to antigen must be compared with controls containing medium without antigen because there is significant variability in baseline (background) immune responses from person to person, and from study animal to study animal [69]. The enzyme-based ELISpot technique is one of the most sensitive methods for detecting T cell responses to therapeutic proteins. It enables the detection of individual T cells directly from splenocytes or peripheral blood and permits determination of their antigen-specific cytokine release. ELISpot assays are regularly used to identify low-frequency autoreactive T cells. IL-2 and IL-4 ELISpot assays can be performed using commercial kits.

Flow cytometry. Fluorescent labeling (FACS) and intracellular cytokine staining (ICS) are the most precise methodologies available for measuring and defining T cell responses because they allow gating of specific types of T cells (including Tregs) but they are associated with a higher cost per assay. For example, T cells that respond to a particular epitope can be directly labeled using tetramers (comprising MHC class II-peptide complexes), or the number and phenotype of T cells that respond to the antigen can be determined using FACS-based assays, cell surface markers, and intracellular cytokine staining [70].

Antigen. The use of whole antigen might not correlate with *in vivo* responses owing to differences in the processing of the protein into its peptide components. Most T cell immunologists substitute overlapping peptides representing the whole antigen, either in pools or in single well assays. There are several inherent problems in the design of overlapping peptides sets for use in T cell assays, which include the artificial truncation of T cell epitopes and the lack of stabilizing flanking residues. Selection of peptides for immunogenicity assays based on epitope-prediction tools is an alternative approach.

only means of discriminating between neutralizing and non-neutralizing ADAs is to perform a biological assay using cell lines that express the target and mimic the therapeutic effect of the drug. Measurement of the biological effects of ADAs was also discussed by Gupta *et al.* in a recent white paper [26].

Prediction of immunogenicity

Several approaches can be used to predict the potential immunogenicity of the therapeutic in a patient, and thereby minimize the risk of adverse reactions or neutralization of the product.

Prediction based on physico-chemical characterization

For non-human proteins, sequence and T cell epitope analysis are good predictors of an immune response in the clinic. For biopharmaceuticals with a high degree of homology to native proteins, the main physico-chemical determinants of immunogenicity are impurities, heterogeneity, aggregate formation, and protein degradation, such as oxidation and deamidation. Aggregate formation is one aspect of the production process that can be monitored by physico-chemical characterization [27]. However, factors such as the formation of repeating arrays might influence immunogenicity and cannot be identified by current physico-chemical methods [28].

Purity

The relatively high immunogenicity of the earliest therapeutic recombinant DNA-derived growth hormone was blamed on high levels of contaminating bacterial products [29]. The considerable reduction in immunogenicity observed over time in products such as insulin and growth hormone is probably due to heightened stringency in production and increased purity. Based on the recent description of the role of TLR ligands in generating a humoral immune response, these products should be removed in the production process, as far as possible [30].

Prediction based on T cell responses

T cell assays can determine whether peptides derived from the therapeutic protein contain sequences with a propensity to stimulate an antibody response. Generally, for protein administered in the context of clinical trials, the best source of T cells for immunogenicity studies is blood obtained from exposed subjects. T cell assays can be

performed with fresh cells *ex vivo* [31], or memory cells can be expanded and activated *in vitro*, if necessary. In both cases, measurement of the T cell response to autologous proteins might require prior depletion of Tregs to unmask weak responses [32]. T cell proliferation, ELISA, or ELISpot without assessment of concurrent Treg responses might give an inaccurate picture of the potential for immunogenicity of autologous proteins. It has been the experience of the authors that assays using blood from exposed individuals are easier to perform and more reliable than assays that use naïve donor cells (A.D.G. and D.S., unpublished results).

HLA-binding assays

HLA-binding assays can be used to assess whether peptides derived from the therapeutic bind to MHC Class II. These assays measure the affinity of predicted epitope sequences for multiple HLA alleles. *In vitro* evaluation of MHC binding can be performed by measuring the ability of exogenously added peptides to compete with a fluorescently-labeled MHC ligand [33]. Competition-based HLA-binding assays can be adapted for high throughput [34].

In silico T cell epitope prediction and content

Several computerized T cell epitope-mapping tools are now available for use in the identification of T cell epitopes contained within protein sequences [35,36]. Highly immunogenic proteins contain many T cell epitopes or concentrated clusters of T cell epitopes, whereas non-immunogenic proteins tend to contain fewer epitopes. One concept, developed by Martin and De Groot, is that T helper (Th) epitope content might explain differences in observed antibody responses to slightly different versions of the same recombinant human protein [37]. Their 'Td immunogenicity scale' enables the evaluation and comparison of protein sequences for T cell epitopes using EpiMatrix (and the matrices for eight common class II alleles). T cell epitope content, as defined for the immunogenicity scale, relates to the number of T cell epitopes per 1,000 amino acids in a protein sequence. For example, monoclonal antibodies that are slight sequence variants of one another can be compared for epitope content (and potentially for immunogenicity), facilitating the process of selecting the best candidate for clinical trials. Epitope prediction is best performed using a combination of the above techniques (*in silico* and *in vitro*). Because *in silico* tools might vary (see [35] for review of epitope-prediction tools), protein developers are advised to request or perform comparisons of prediction accuracy before selecting one T cell epitope prediction tool over another.

Animal studies

In principle, all proteins intended for therapeutic use in humans are foreign for animals, and therefore immunogenic. Animal and human responses to proteins of non-human origin will be similar because these proteins are comparably foreign for all mammalian species. Although the use of the respective animal proteins might help elucidate basic immunological properties of the molecules studied, animal and human MHC are not equivalent because they can have different amino acid side-chain restrictions. This is best illustrated by the variability of immune response to chimeric antibodies in different strains of inbred mice [38]. For human homologs, standard animal models are even less predictive because the induction of immunogenicity in humans is based on breaking B and T cell tolerance, although such models do allow comparison of relative immunogenicity of different preparations of the same protein. In non-human primates, there might be a higher degree of sequence homology between the human product and the primate native molecule, to which the animal is immune tolerant. However, one should be aware of significant differences in immunogenicity between monkeys and humans. For example, rhesus monkeys (*Macaca mulatta*) and cynomolgus monkeys (*Macaca fascicularis*) respond differently to human EPO–GM-CSF hybrids, whereas human FVIII at therapeutic doses can be immunogenic in rhesus monkeys (D.S. *et al.*, unpublished results). Another means of evaluating the impact of epitope modifications on *de novo* T cell response is to measure immunogenicity of the proteins and their derivative peptide epitopes in HLA-transgenic mice [39]. Fortunately, several transgenic mouse strains expressing the most common HLA DR molecules are available [40,41]. To compare immunogenicity of wild-type and modified epitopes, mice are immunized with peptide epitopes in adjuvant. T cell responses in infected humans correlate directly with T cell responses in immunized HLA transgenic mice [42,43]. Hence, HLA transgenic mice are now routinely used to assay and optimize (human) epitope-driven vaccines in pre-clinical studies [44–46].

Protein-transgenic mice for predicting breakage of B cell tolerance

An alternative model for the evaluation of immunogenicity of autologous human proteins is a transgenic animal expressing the human protein in question, which is therefore tolerant to it. Breaking of immune tolerance can be studied in these animals as reported by Ottesen *et al.* [47], Palleroni *et al.* [48] and Stewart *et al.* [49] for insulin, IFNα2 and human-tissue plasminogen activator (htPA), respectively. Such transgenic mice were used to show that aggregates of human IFNα2 produced during storage at room temperature of freeze-dried preparations were responsible for enhanced immunogenicity [50].

Reduction of immunogenicity: therapeutic protein modification

Protein therapeutic developers aiming to decrease potential immunogenicity of their products have several options available: careful formulation (to avoid contaminants and aggregates), reduction of immunogenicity by glycosylation and PEGylation, and, in some cases, linkage of the therapeutic protein to a human Ig Fc fragment. Reduction of T cell epitopes by sequence modification also improves immunogenicity, as described below.

PEGylation and glycosylation

PEGylation and glycosylation are the two best known methods for post-translational modification of proteins that impact therapeutic efficacy. PEG is non-toxic, non-immunogenic, non-antigenic, highly soluble in water and

has US FDA approval for use in a range of therapeutics. PEGylated therapeutic protein conjugates have several advantages, including decreased degradation by metabolic enzymes and prolonged residence time in the body. In addition to prolonging protein half-life, PEG might decrease immunogenicity by decreasing proteolysis, interfering with protein processing and presentation, and by physically blocking antibody- or HLA-epitope binding [51]. Glycosylation is also believed to interfere with antibody binding and to have an impact on autoimmunity [52]. The protein-processing machinery of APCs might be impeded by glycosylated amino acid residues and, alternatively, peptide processing might modify (partially or fully deglycosylated) sugars, potentially affecting epitope presentation to T cells [53]. A glycan-containing T cell epitope might also be presented as several variants. This might impair or diversify the immune response by interfering with the binding of the epitope to HLAs or by modifying the interaction with the TCR. Larger glycan structures can lead to loss of MHC class II-restricted T cell recognition.

IgG fusion proteins

Isologous and heterologous immunoglobulins are extremely effective as carriers of therapeutic moieties. The efficacy of these proteins is due, in part, to their long half-life *in vivo*, as well as their ability to bind to Fc receptors. The concept of using IgG as a carrier molecule to induce unresponsiveness in the adult immune system has been exploited for simple haptens, such as nucleosides, as well as for peptides [54]. The most recent example of a successful effector–Fc fusion is etanercept (Enbrel) (Amgen Inc.; http://www.amgen.com/), a fusion protein between soluble TNF receptor Type II and the Fc region of human IgG1, which has been used in clinical practice for the control of a wide range of chronic inflammatory diseases, such as rheumatoid arthritis.

Humanization

Much effort has been applied to re-engineering murine and mouse–human chimeric antibodies so that the antibodies have as little non-human structure as possible, to minimize anti-therapeutic effects. In some cases, modification of the therapeutic protein sequence from a foreign protein (a murine monoclonal) to a less foreign protein (a humanized monoclonal) has resulted in reduced immunogenicity. Replacement of mouse immunoglobulin constant regions with human sequences results in the largest immunogenicity reduction. Humanization of variable domains affects a further decrease. Although there is clear evidence that chimeric antibodies are less immunogenic than murine monoclonal antibodies, humanization has not solved the monoclonal antibody immunogenicity problem. For example, the humanization approach reduced, but did not entirely eliminate, the immunogenicity of the Campath antibody [55,56].

Sequence modification

Because the T cell epitope has a crucial role in the development of Td antibody responses, it stands to reason that protein-sequence modifications resulting in removal of potential T cell epitopes from autologous (recombinant therapeutic) proteins could reduce potential for the induction of immune response to the protein. The literature is replete with evidence for the attenuating effect of epitope-sequence modification on T cell response, particularly with reference to immune escape from class I- and class II-restricted immune response in viral infections. Loss of T cell help removes Signal 2 for specific B cells and, in theory, could lead to B cell tolerance [57]. T cell epitope modification might be thought of as the directed version of the process that occurs naturally when tumor cells [58] and pathogens [59,60] evolve to escape immune pressure by accumulating mutations that reduce the binding of their constituent epitopes to host HLAs [61]. This approach might be relevant to the design of novel replacement proteins, as illustrated by the development of a deimmunized rEPO [62] and recent progress on a novel FVIII [63], and by the development of deimmunized monoclonal antibodies [64–66]. Successful deimmunization of therapeutic proteins has been demonstrated by Genencor Inc. (http://www.genencor.com) [67] and EpiVax Inc. (http://www.epivax.com) [68] using slightly different approaches. However, such modification could introduce new epitopes that could be presented by other MHC molecules or could create novel B cell epitopes – possibilities that can be assessed in (HLA transgenic) animal studies or by reiterative T cell epitope analysis (*in silico*). Finally, these modifications must maintain the biological function of the product.

Conclusion: is there a bright new future?

Given the vast array of therapeutic proteins that are entering the clinic, all researchers and developers involved in the production of therapeutic proteins should consider the potential for their product to induce an immune response in humans, and should carry out careful immunogenicity studies in the preclinical and clinical phases of the development program. They might also add T cell epitope mapping and screening studies in suitable animal models to their pre-clinical repertoire, before moving a promising candidate into the clinic. In addition to T cell epitope mapping, a careful evaluation of the phenotype of the type of T cells responding to the study protein is warranted because newer data are now demonstrating that not all T cell 'responses' are equivalent. This holds true particularly for proteins that are in part, or wholly, recombinant autologous proteins, such as FVIII or any of the cytokines. Although these proteins might contain multiple epitopes, the T cells that respond to the epitopes might be effector or regulatory in nature.

Research on the nature of the immune response to autologous (homologous) therapeutic proteins could improve the selection and screening of candidates. It should build on information about the innate immune system, B cell signaling and tolerance, the importance of Treg, and the regulation of autoimmunity in the periphery. Whatever the 'final' mechanism might be, it is important to remember that the goal of this research is to accelerate the development of new compounds that are safe for use in human subjects. The bright new future of therapeutic proteins will arrive when, and if, developers remember to consider immunogenicity.

Conflict of interest statement

Dr. Annie De Groot is a majority stockholder in EpiVax and an employee of EpiVax.

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